Defense Threat Reduction Agency
8725 John J. Kingman Road, MS
6201 Fort Belvoir, VA 22060-6201

DTRA-TR-15-48

TECHNICAL REPORT

Novel Formulation to Destroy Biothreat Agents

February 2016

HDTRA1-11-1-0051

Adam Driks and Derrell McPherson

Prepared by:
Loyola University Chicago
2160 S. 1st Avenue
Maywood, IL 60153

DESTRUCTION NOTICE:

Destroy this report when it is no longer needed.
Do not return to sender.

PLEASE NOTIFY THE DEFENSE THREAT REDUCTION AGENCY, ATTN: DTRIAC/ J9STT, 8725 JOHN J. KINGMAN ROAD, MS-6201, FT BELVOIR, VA 22060-6201, IF YOUR ADDRESS IS INCORRECT, IF YOU WISH IT DELETED FROM THE DISTRIBUTION LIST, OR IF THE ADDRESSEE IS NO LONGER EMPLOYED BY YOUR ORGANIZATION.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
00-02-2016	Technical	August 29, 2011 - March 6, 2015

4. TITLE AND SUBTITLE

Novel Formulation to Destroy Biothreat Agents

5a. CONTRACT NUMBER

HDTRA 1-11-1-0051

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Adam Driks (LU)
Derrell McPherson (NSWC)

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Loyola University Chicago
2160 S. 1st Avenue
Maywood, IL 60153

Naval Surface Warfare Center, Dahlgren Division
4045 Higley Road, Suite 345
Dahlgren, VA 22448

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Defense Threat Reduction Agency
8725 John J. Kingman Road, STOP 6201
Fort Belvoir, VA 22060-6201

10. SPONSOR/MONITOR'S ACRONYM(S)

DTRA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

DTRA-TR-15-48

12. DISTRIBUTION / AVAILABILITY STATEMENT

Distribution Statement A. Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this work was identification of gentle chemistries, and degrative enzyme formulations, that can be formulated to efficiently destroy Bacillus anthracis spores. To achieve this, the effects on spore morphology and viability of a diverse range of chemicals and enzymes were analyzed. It was found that spores are remarkably resistant to even relatively reactive chemistries. As a result, no mild, sporocidal chemistry was identified. These results identify novel chemical resistance properties of the spore. A formulation containing the enzyme papain that readily reduced spore viability by over 5 logs was identified. Importantly, for maximum effect, the formulation required the inclusion of germinants. This did not cause full outgrowth and, therefore, did not result in complete removal of the spore's protective shells. However, it did result in a sufficient breach in these shells to permit efficient degradation by papain, which does not take place when the exosporium is intact. These results not only identify an effective sporocidal formulation, but also provide novel information regarding the roles of spore structures in resistance and the mechanism of spore germination.

15. SUBJECT TERMS

Bacterial spore, decontamination, coat, exosporium, bacillus anthracis spores

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	43	Heather Meeks
Unclassified	Unclassified	Unclassified			**19b. TELEPHONE NUMBER** *(include area code)* 703-767-3059

UNIT CONVERSION TABLE

U.S. customary units to and from international units of measurement*

U.S. Customary Units	Multiply by → / ← Divide by†		International Units
Length/Area/Volume			
inch (in)	2.54	$\times 10^{-2}$	meter (m)
foot (ft)	3.048	$\times 10^{-1}$	meter (m)
yard (yd)	9.144	$\times 10^{-1}$	meter (m)
mile (mi, international)	1.609 344	$\times 10^{3}$	meter (m)
mile (nmi, nautical, U.S.)	1.852	$\times 10^{3}$	meter (m)
barn (b)	1	$\times 10^{-28}$	square meter (m^2)
gallon (gal, U.S. liquid)	3.785 412	$\times 10^{-3}$	cubic meter (m^3)
cubic foot (ft^3)	2.831 685	$\times 10^{-2}$	cubic meter (m^3)
Mass/Density			
pound (lb)	4.535 924	$\times 10^{-1}$	kilogram (kg)
unified atomic mass unit (amu)	1.660 539	$\times 10^{-27}$	kilogram (kg)
pound-mass per cubic foot (lb ft^{-3})	1.601 846	$\times 10^{1}$	kilogram per cubic meter (kg m^{-3})
pound-force (lbf avoirdupois)	4.448 222		newton (N)
Energy/Work/Power			
electron volt (eV)	1.602 177	$\times 10^{-19}$	joule (J)
erg	1	$\times 10^{-7}$	joule (J)
kiloton (kt) (TNT equivalent)	4.184	$\times 10^{12}$	joule (J)
British thermal unit (Btu) (thermochemical)	1.054 350	$\times 10^{3}$	joule (J)
foot-pound-force (ft lbf)	1.355 818		joule (J)
calorie (cal) (thermochemical)	4.184		joule (J)
Pressure			
atmosphere (atm)	1.013 250	$\times 10^{5}$	pascal (Pa)
pound force per square inch (psi)	6.984 757	$\times 10^{3}$	pascal (Pa)
Temperature			
degree Fahrenheit (^oF)	$[T(^oF) - 32]/1.8$		degree Celsius (^oC)
degree Fahrenheit (^oF)	$[T(^oF) + 459.67]/1.8$		kelvin (K)
Radiation			
curie (Ci) [activity of radionuclides]	3.7	$\times 10^{10}$	per second (s^{-1}) [becquerel (Bq)]
roentgen (R) [air exposure]	2.579 760	$\times 10^{-4}$	coulomb per kilogram (C kg^{-1})
rad [absorbed dose]	1	$\times 10^{-2}$	joule per kilogram (J kg^{-1}) [gray (Gy)]
rem [equivalent and effective dose]	1	$\times 10^{-2}$	joule per kilogram (J kg^{-1}) [sievert (Sv)]

*Specific details regarding the implementation of SI units may be viewed at http://www.bipm.org/en/si/.

†Multiply the U.S. customary unit by the factor to get the international unit. Divide the international unit by the factor to get the U.S. customary unit.

Final Report

Dates covered: Nov. 2011 – January. 2015

Novel Formulation to Destroy Biothreat Agents

Submitted by:

Adam Driks, Ph.D.
Loyola University Chicago
2160 S. 1st Avenue
Maywood, IL 60153
Telephone: (708) 216-3706
Email: adriks@lumc.edu

Derrell McPherson, Ph.D.
Naval Surface Warfare Center, Dahlgren Division
CBR Concepts & Experimentation, Code Z21
4045 Higley Road, Suite 345
Dahlgren, VA 22448
Telephone: (540) 653-2080
Email: derrell.mcpherson@navy.mil

Submitted to:
DTRA Basic Science
Dr. Heather Meeks

Date:
January 19th, 2015
Capability Area: Agent Defeat
Period of Performance: FY11-FY15

1. PROJECT RATIONALE

This project is a basic science investigation into novel methods to render *Bacillus anthracis* spores sensitive to standard, relatively gentle decontamination technologies. This project is motivated by the highly resistant nature of *Bacillus anthracis* spores. Because this extreme level of resistance renders the spore insensitive to all but very harsh decontaminants, and since harsh treatments are impractical in many scenarios, there is a need for methods that make spores susceptible to relatively mild, standard decontamination. Unfortunately, historically this has been a very challenging problem. *Bacillus anthracis* spores appear to be resistant to diverse assaults and the literature did not contain clear paths forward. Therefore, a basic science investigation to identify treatments rendering spores susceptible to standard mild decontamination was required. Our overarching hypothesis is that the spore coat and/or exosporium are the major protective structures resisting mild decontamination (Fig. 1). We further hypothesized that 1) as yet unidentified chemistries can be used to induce at least partial disassembly of these structures and/or 2) enzymes, and in particular proteases, can be used to create breaches in these structures. If successful, either of these treatments alone, or combined into a novel formulation, would be expected to leave spores in a condition in which they would be susceptible to a mild decontaminant.

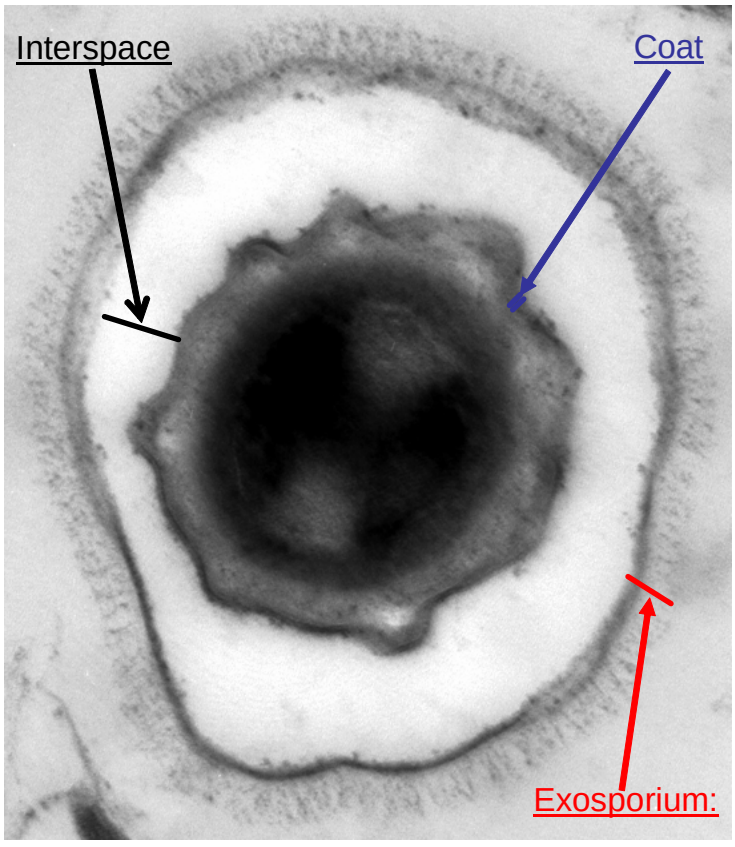

Figure 1. Electron micrograph of a *Bacillus anthracis* (Sterne strain) spore.

The initial goals of this work involved a broad search among diverse chemistries and enzymes for those that damage *B. anthracis* spores. Overall, the effort was to identify 1) gentle chemistries and 2) enzymatic treatments that will render spores susceptible to relatively mild decontamination. It was hypothesized that to do this, these treatments must, at a minimum, damage the coat and/or exosporium. At the start of the work, no such chemistries or enzymes were known, and no obvious candidates were available. Therefore, in practice there were no obvious limitations to our initial choices of chemistries or enzymes to analyze.

2. EXPERIMENTAL RESULTS

A. Analysis of the effects of diverse chemistries on spore integrity and function.

The biochemical basis of the molecular interactions responsible for the assembly and maintenance of the exosporium, coat, and other spore layers remains largely unknown. The goal of this topic is to identify gentle chemistries that would attack and disrupt essential, yet not understood chemistries that hold those protective structures together. Breaking open the coat and other structures might allow a mild decontaminating reagent to penetrate to the core and destroy the macromolecules required for metabolic activity after germination. This would result in killing the spore. To begin to identify chemistries that can damage spore structures, reagents from a diverse set of chemical classes were tested. Initially, these reagents were used under relatively harsh conditions that would maximize their effect on the spore and, therefore, most easily identify them as candidates. It was reasoned that if the approach was successful in this initial test, the approach could then be modified to analyze these chemicals under more gentle conditions. The primary approach was to examine the spore by transmission electron microscopy (TEM) to determine whether any structural damage could be observed. The spores that did show structural damage were also tested for increased susceptibility against various treatments that spores are typically resistant. These will be discussed below.

Throughout this study, over 50 chemicals from a variety of classes were tested to see if any specific chemical reaction might sufficiently disrupt interactions among the macromolecules causing the structures to break open. These classes included: acids, amine oxides, aromatic/organic compounds, chaotropic agents, copper compounds, ionic liquids, oxidizing agents, reducing agents, salts, and surfactants. The goal for treating spores with the variety of chemical categories (Table 1) was to identify one or more classes that created a gap in either the coat or the exosporium. This gap might be quite small (<0.0001 mm) and, therefore, would require high resolution analysis, such as TEM. Of all the classes tested, the acids and reducing agents caused the most significant effects. Interestingly, even if some chemicals of a class caused significant damage, others of the same class would result in no visible damage. In fact, most reagents produced no visible change in spore ultrastructure. In brief summary, it was found that only a subset of reagents within most chemical categories caused changes in at least one spore structure (Table 1):

Table 1. Summary of Chemical Treatments on the Spore Structures of *B. anthracis* Sterne [a]

Chemistry (concentration)	**Structure affected by reagent**		
	Exosporium	**Coat**	**Cortex**
Controls			
Water	No	No	No
Ethanol (95%)	No	No	No
Acids			
Acetic acid (0.5 M)[b]	No	**Yes**	No
Hydrochloric acid (0.5 M)[b]	**Yes**	**Yes**	**Yes**
Hydroiodic acid (0.5 M)[b]	No	**Yes**	**Yes**
Iodic acid (0.5 M) [b]	No	No	No
Nitric acid (0.5 M)[b]	No	**Yes**[f]	**Yes**
Sulfuric acid (0.5 M)[b]	No	No	No
Amine Oxides			
,N-dimethyldecylamine N-oxide (Barlox™ 10S) (1 M, 24 hr at 45°C)[b]	No	No	No
(3-Phenylpropyl) pyridine N-oxide (1 M, 24 hr at 45°C)[c]	No	No	No
Aromatics and Organics			
Acetone (neat)	No	No	No
Benzophone (1 M)[d]	No	No	No
Benzoic acid (1 M)[d]	No	No	No
Benzyl alcohol (neat)	No	No	No
2,5-Dichlorophenol (1 M)[d]	No	No	**Yes**
2,6-Dihydroxynapthalene (1 M)[d]	No	No	No
N,N-dimethylformamide (neat)	No	**Yes**	No
1-Phenyl-1-2,-ethanediol (1 M)[d]	No	No	No
2-Phenoxyethanol (neat)	No	**Yes**	No
1-Phenoxy-2-propanol (neat)	No	No	**Yes**
Chaotropic Agents			
Ammonium sulfate (1 M)[b]	No	No	No
Urea (1 M and 6 M)[b]	No	No	No
Copper Compounds			
Copper (II) acetate (1 M)[b]	No	No	No
Copper (II) aceylacetonate (0.5 M)[b]	No	No	No
Copper (II) chloride (1 M)[b]	No	No	No
Ionic Liquids			
1-butyl-3-methylimidazolium octyl sulfate (1 M)[c]	No	No	No
1-butyl-3-methylimidazolium thiocyanate (1 M)[c]	No	No	No
ethyl-3-methylimidazolium 1,1,2,2-tetrafluoroethanesulfonate (1 M)[c]	No	**Yes**	No
ethyl-3- methylimidazolium bis(trifluorosulfonyl)imide (1 M)[c]	No	**Yes**	No
1-ethyl-3-methylimidazolium dicyanamide (1 M)[c]	No	No	No
hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide (1 M)[c]	No	No	No
hexyl-3-methylimidazolium tetraefluoroborate (1 M) [c]	No	No	No

1-methyl-3-methylimidazolium thiocyanate (1 M)[c]	No	No	No
ethyl-trioctylammonium bis(trifluoromethylsulfonyl)imide (1 M)[d]	No	No	No
Oxidizers			
Peracetyl borate (PES-Solid) alone (170 mg/mL)[b]	**Yes**	No	No
racetyl borate (PES-Solid) Dahlgren Decon™ (170 mg/mL)[b]	Yes	**Yes**	**Yes**
Sodium percarbonate (10 mg/mL, 60 min at 65°C)[b]	No	No	No
Reducing Agents			
1,4-Benzenedithiol (1 M)[d]	**Yes**	**Yes**	**Yes**
Benzyl mercaptan (neat)	**Yes**	**Yes**	**Yes**
Dithiothretiol (0.001 M - 0.5 M, 24 hr at 45°C)[b]	**Yes**	No	No
4-Mercaptobenzoic acid (1 M)[d]	No	No	No
2-Mercaptoethanol (1 M)[b]	No	No	No
Thiourea (1 M and 3 M)[b]	No	**Yes**	**Yes**
is (2-carboxylethyl) phosphine hydrochloride (TCEP) (0.5 M)[e]	No	No	No
Sodium sulfite (1 M)[b]	No	No	No
Sodium thiosulfate (5 % w/v)[b]	No	**Yes**	No
Sodium thioglycolate (1 M)[b]	No	No	No
Salts			
NaCl (1 M and 6 M)[b]	No	No	No
$MgCl_2$ (1M and 6 M)[b]	No	No	No
Surfactants			
Dodecyltrimethylammonium chloride (1 % w/v)[e]	No	No	No
hoxylated fatty alcohol (Synperonic™ 13/6-LQ-(AP)) (1M)[b]	No	No	No
Sodium decyl sulfate (1 % w/v)[e]	No	No	No
Sodium dodecyl sulfate (1 % w/v)[b]	No	**Yes**[f]	**Yes**
Sodium tetradecyl sulfate (1 % w/v)[e]	No	No	No
Triton X-100 (1 % w/v)[b]	No	No	No

[a] All reactions were done at room temperature for 60 min unless otherwise noted.
[b] Dissolved in water.
[c] Dissolved in 70% Ethanol.
[d] Dissolved in 95% Ethanol.
[e] Dissolved in 50 mM HEPES.
[f] The coat is damaged in these images, however it is unclear if the coat is broken or only separated from the cortex as a result of core leakage.

1. Identification of chemistry classes that alter spore structure.

For each chemical, 10^9-10^{11} *B. anthracis* Sterne spores were treated with 1mL of each chemical solution. The spores were typically treated with each chemical for 1 hour at room temperature. However, some chemicals were more reactive at higher temperatures and/or required longer times. For instance, sodium percarbonate required 65°C for 60 min for activation and the amine oxides required 45°C for up to 24 hours for optimal reactivity. These amine oxide conditions were initially used for dithiotretiol (DTT) since the experiments were run concomitantly,

although follow-on tests with DTT examined the effect with shorter times and lower temperatures. After incubation, the spores were rinsed with water, a small aliquot of each sample was tested for viability and the remaining sample was processed for TEM to assess the coat, cortex, and exosporium for damage. The chemicals that cause changes to the spore structures were also tested for viability against mild decontamination techniques that spores would normally be resistant to. These treatments included: heat (65^{o}C for 30 min), lysozyme (0.5- 25 mg/mL at 37^{o}C for 15 min), and ethanol (70% or 100% for 15 min).

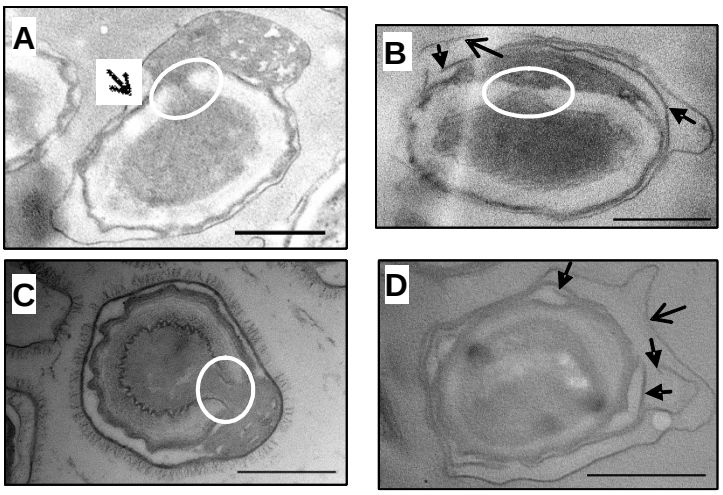

Figure 2. Electron micrographs of *B. anthracis* Sterne spores treated with various acid chemicals. Spores were treated with either 0.5 M nitric acid (A), 0.5 M hydrochloric acid (B), 0.5 M hydriodic acid (C), and 0.5 M acetic acid (D) as described in text. Spore structures are identified by line arrow (exosporium) or black arrows (coat). Ruptures in the cortex are circled. Size bars = 500 nm.

As the interactions found in the coat are currently so poorly understood, the results are ambiguous. However, the researcher's hypotheses developed from observed results based on the knowledge of the chemical properties of each reagent. Only the chemicals with structural affects are described below according to their chemical classes.

a. Acids: Acids by definition have at least one hydrogen atom that they can easily release to other surrounding compounds. Acids with varying pKa's were chosen to see if protonation would affect spore structure stability. Protonation can result in altered charged states of the proteins or other species found on the spore altering ionic interactions and other Van der Waals forces holding these structures together. Strong acids can also break bonds by hydrolyzing proteins and sugar molecules, which can damage the integrity of the spore structures whose components are largely comprised of such macromolecules.

Most acids had a significant effect on the spore structure and viability. Nitric acid, hydrocholoric acid, and hydriodic acid all caused the cortex and inner membrane to rupture and the core to leak out (Fig. 2A-C). Hydriodic acid also broke the coat (Fig. 2C). Hydrocholoric acid caused the coat and exosporium to have breaks and the coat to be separated from the cortex (Fig. 2B). Acetic acid caused a loosening and possibly coat breakage, but the exosporium remained intact (Fig. 2D). Despite some strong acids showing large structural damage, two strong acids (sulfuric acid and iodic acid) showed no structural damage. The reason for this is unknown. Acids had the greatest effect on spore viability, even acids which causes no visual damage still resulted in some loss in viability. The log reduction in spore

viability was as follows: hydrochloric acid, 3 logs; nitric acid, 4 logs; acetic acid, 2 logs; and sulfuric acid, 1 log.

b. Aromatic and Organic Compounds: A series of aromatic and organic compounds of varying levels of hydrophobicity were tested. These compounds were chosen with the idea that they would be able to form non-covalent interactions with the hydrophobic groups in the spore coat/exosporium and potentially break those natural interactions.

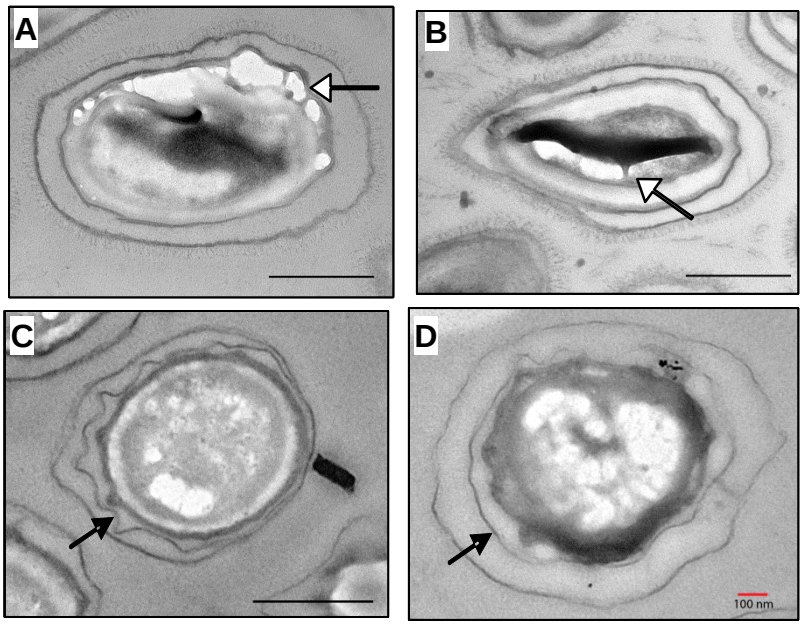

Figure 3. Electron micrographs of *B. anthracis* Sterne spores treated with various aromatic and organic chemicals. Spores were treated with 1M 2,5-Dicholorphenol (A), neat 1-Phenoxy-2-propanol (B), neat N,N-dimethylformamide (C), and neat 2-phenoxyethanol (D) as described in text. The first two chemicals caused damage to the cortex is shown with white arrowheads, while the second two caused damage to the coat shown with black arrowheads. Size bars are 500 nm for A-C and 100 nm for D.

Only a couple chemicals caused disruptions in this series. 2,5-Dichlorophenol and 1-phenoxy-2-propanol showed a disruption between the cortex and core interactions (Fig. 3A, B). These changes were minor and suggest that the affects were minimal. N,N-dimethylformamide and 2-phenoxyethanol showed separation of a thin layer of the outer coat from the larger portion of the coat material that remained adjacent to the cortex (Fig. 3C, D). All of these chemicals did not show any breaks in the coat, cortex, or exosporium. This suggests that some hydrophobic interactions do exist, possibly more so between the structural layers, but the interactions appear to be selective as not all the reagents caused an effect. None of these chemicals showed an effect on spore viability.

c. Ionic Liquids: Ionic liquids are salts with a melting point near room temperature. They are typically comprised of one or more organic ions with a sterically shielded ionic core, preventing close association of ion pairs in the salt. Since they are ionic, they dissolve a number of organic and inorganic ions as well as polar organic molecules. The interaction of ionic liquids with the coat could aid in the dissociation of charged species present within the coat.

Only two compounds caused disruptions. These two ionic liquids (1-ethyl-3-methylimidazolium 1, 1, 2, 2-tetrafluoroethanesulfonate and 1-ethyl-2-methylimidazolium

bis(trifluoromethylsulfonyl)imide) caused the coat to break off in shards in ≤ 50% of the spores (Fig. 4A, B). The shards are likely part of the outer coat layers since it also appears that unbroken coat material is still adjacent to the cortex. This again shows how the interactions within the coat may be specialized and selective, as only 2 of the 9 chemicals tested had any effect. There was no loss in spore viability with these chemicals.

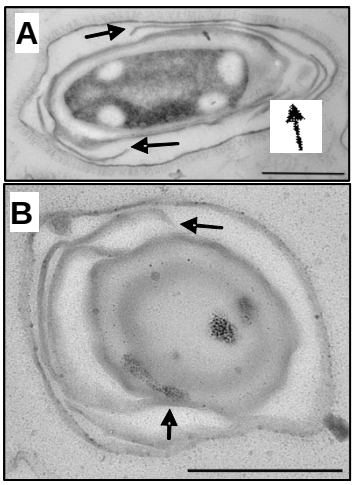

Figure 4. Electron micrographs of *B. anthracis* Sterne spores treated with two ionic liquids. Spores were treated with 1 M 1-ethyl- 3-methylimidazolium 1,1,2,2-tetrafluoroethansulfonate (A) and 1 M 1-ethyl- 3-methylimidazolium bis(trifluoromethylsulfonyl)imide (B) as described in text. Separation and breakage of the coat from the cortex is shown with the black arrows. Size bars = 500 nm.

d. Oxidizing agents: Oxidizing agents can readily accept electrons from various reducing agents. Peractic acid (PES-solid) and sodium percarbonate are both oxidizing agents and can form radical species. Sodium percarbonate must be warmed above its dissociation temperature to become activated.

Oxidizing agents were shown to greatly alter spore structure. Peracetic acid alone appears to dramatically damage most spores, causing them to lose their rigid, oval or round structure and become more pliable; allowing the sides to appear flattened (Fig. 5A). Also the exosporium appears to be absent in all spores, though the coat and cortex appear intact. This phenotype is more extreme after treatment with Dahlgren Decon™, where the cortex is absent in most spores and the structural integrity of those spores is lost, resulting in highly irregular-shaped spores (Fig. 5B). Strikingly, all the structural detail is lost in

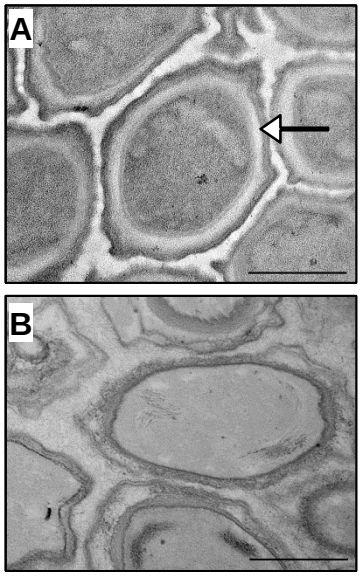

Figure 5. Electron microscopy of *B. anthracis* Sterne spores treated with oxidizing agents. Spores were treated with 170 mg/mL peracetic acid (PES) alone (A) or as a component of the Dahlgren Decon™ formulation (B) as described in text. In (A) spores lack the exosporium and have damage to the cortex (white arrowhead) which resulted in loss of spore rigidity and core hydration. In (B), spores have at least the degree of damage seen in (A) but with the total loss of cortex, and therefore, loss of normal spore shape. Size bars = 500 nm.

the region of the image inside the boundary of the coat. However, a few spores still retain a largely normal shape and apparently intact core and cortexes. In these cases, the coat appears to have separated from the cortex and into layers. The exosporium is absent from all the spores in the images. Both PES-solid alone and Dahlgren Decon™ formulation caused a loss of viability. PES-solid alone resulted in a 3 log kill and the Dahlgren Decon™ resulted in a 4-5 log kill.

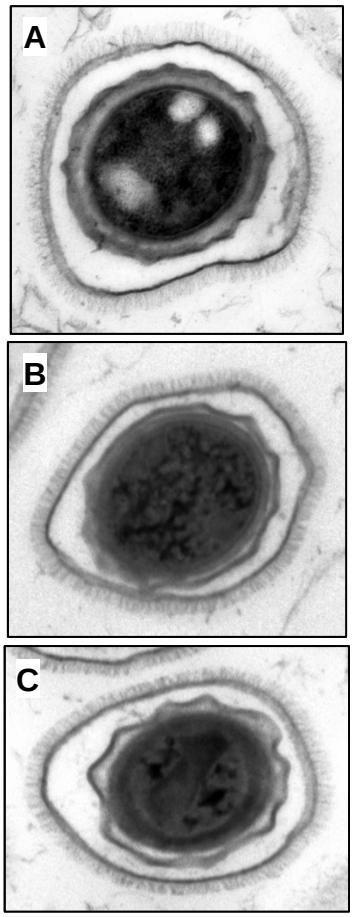

Figure 6. Effect of sodium percarbonate treatment on *B. anthracis* Sterne spores. An untreated spore (A) or spores reacted with sodium percarbonate (B,C) (as described in the text) were imaged by thin-section electron microscopy. Treatment results in a significant change in core morphology in which the usual punctate structures (ribosomes) and clear regions (containing DNA) are no longer apparent; instead the core has a uniformly mottled appearance.

Sodium percarbonate treatment does not detectably alter exosporium or coat structure (Fig. 6). However, it has a significant effect on the core. Instead of the usually readily detected whitish regions, where DNA is located, or punctate structures, due to ribosomes, the core has a uniformly mottled appearance. This suggests that sodium percarbonate significantly affects core structure and, most likely, function. This is verified as sodium percarbonate caused a 4 log kill.

e. Reducing agents: Reducing agents donate electrons to other species. Reducing agents are highly nucleophilic and could potentially break any peptide disulfide bonds found in the coat interior. They also can reduce sugar molecules breaking oligosaccharides.
Several reducing agents affected the spore structure. The reducing agent dithiothreitol (DTT) caused a break at one pole in the exosporium when reacted at high concentrations (0.5 M) at high temperature (45^{o}C) for a minimum of 24 hours (Fig. 7B). However, no effect was seen at lower temperature, lower times, and lower concentrations. This breakage lead to several questions especially regarding if the breakage occurred at the same pole where the spore naturally breaks during germination. This became a difficult question to answer and after many trials the data was inconclusive (Data not shown).

Other reducing agents caused breakage of the spore structures at different locations. In some spores treated with sodium thiosulfate (STS), at least part of the coat was separated from the cortex and was broken in several places (Fig. 7C). Thiourea had also been shown to affect the coat. Originally the researchers thought it was reacting as a chaotropic agent; however, they now believe thiourea is acting as a reducing agent similar to DTT and STS, since it also has a highly

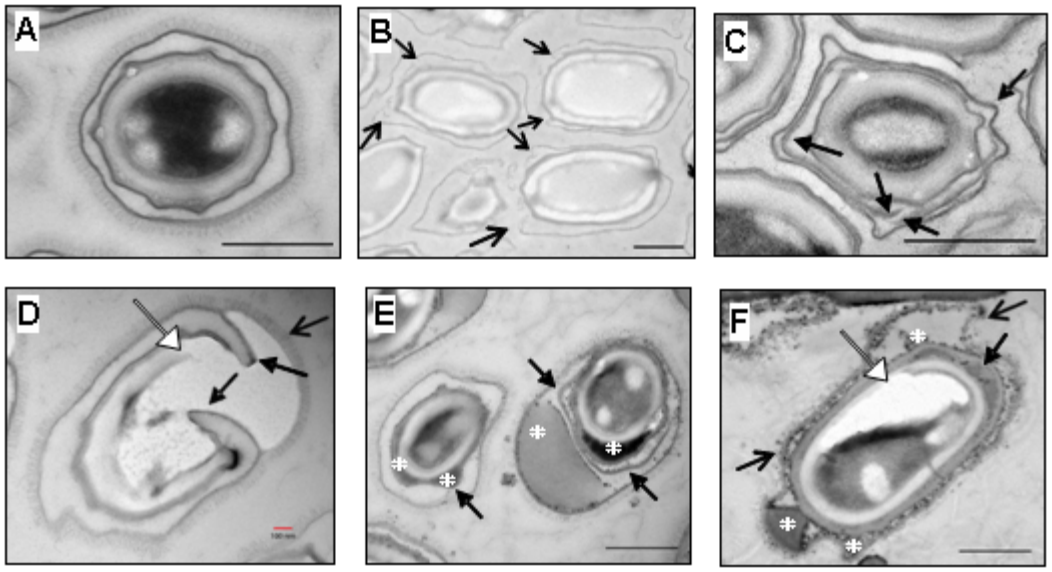

Figure 7. Electron micrographs of *B. anthracis* Sterne spores treated with reducing agents. Spores were untreated (A) or treated with, 0.5 M dithiothreitol (DTT) (B), 5% w/v sodium thiosulfate (STS) (C), 1 M thiourea (D), 1 M benzyl mercaptan (E), and 1 M 1,4 benzenedithiol (F) as described in the text. Spores treated with DTT show a break at one pole of the exosporium (line arrows). Spores treated with STS showed a separation and breakage of the coat (black arrowheads). Thiourea treatment resulted in breakage of the coat (black arrowheads) and cortex (white arrowheads), however the exosporium remains intact (line arrow). Benzyl mercaptan resulted in separation between the layers (black arrowheads) and pools of material forming (white asterisks). Spores treated with 1, 4-benzenedithiol resulted in damage to the coat (black arrowheads), cortex (white arrowhead), and exosporium (line arrow). Pools of material also formed (white asterisks). Size bars are 500 nm for A-C, E, F and 100 nm for D.

nucleophilic sulfur group. Furthermore, >50% of the spores examined show breaks in the coat and approximately 10% had complete breaks in both the coat and cortex (Fig. 7D). The different locations of breakage between DTT (exosporium), STS (coat), and thiourea (coat/cortex) could be due to their different redox potentials and ability of the compound to pass through the layers.

Two additional reducing compounds with highly nucleophilic sulfur centers were found to affect the coat. Both benzyl mercaptan and 1, 4-benzendithiol showed very similar results with separations between each layer and pools of material formed in between each of the layers (Fig. 7E, F). 1, 4-Benzenedithiol also showed a darkening occurring on the outer surface of the exosporium. These features indicate that damage occurred throughout the coat, cortex, and exosporium and were widespread in the sample. The researchers hypothesize that this damage is a result of the nucleophilic sulfur groups breaking the interactions between the layers by reduction. These compounds may be able to penetrate different regions due to their

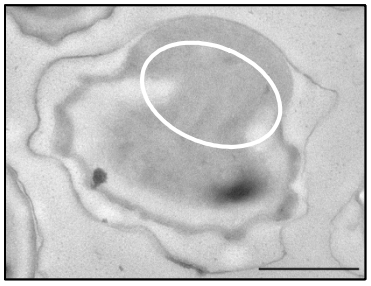

Figure 8. Electron micrograph of *B. anthracis* Sterne spores treated with sodium dodecylsulfate (SDS). Treatment of spores with 1% w/v SDS as described in text resulted in breakage of the cortex and spore membrane causing the spore contents to spill in the space between the coat and exosporium (white circle). Size bar = 500 nm.

hydrophobic properties resulting in a morphological change different than the DTT, STS, or thiourea. Notably the organic and aromatic compounds also saw loosening of the cortex and coat. This data suggests the compounds may be attracted to certain areas of the spore due to their hydrophobicity.

The reducing agents caused the most interesting differences in the spore morphology. It has also been hypothesized that several disulfide bonds play a role in the formation and structure of the spore exosporium, coat, and cortex. The different polarities, molecular size, and redox potentials of all the chemicals may be the reason that different reducing agents affect different layers, as it was observed. Notably while several morphological changes occurred, no changes in viability were seen for any of these compounds suggesting these disulfide bonds are not critical for spore survival. It was also tested if these compounds caused an increased susceptibility to heat, lysozyme or ethanol treatments, although none was found.

f. Surfactants: Surfactants can break apart hydrophobic interactions by solubilizing them in a hydrophilic solution. The reagents may be able to disrupt hydrophobic interactions, especially within membranes which are composed of fatty acids.

Only sodium dodecyl sulfate (SDS) affected spore structure. SDS was successful in rupturing the cortex and core membrane causing the core to leak through the cortex into the interspace (Fig. 8). The exosporium still appears to be intact. Triton X-100 and ethoxylated fatty alcohol did not cause any visible structural damage. However both SDS and Triton X-100 caused a 1 log decrease in spore viability.

g. Chemicals with no Effect: Four other classes of chemicals were tested which had no structural changes or changes in viability. These included amine oxides, chaotropic agents, copper compounds, and salts. Each of these classes has distinct properties and therefore had different possible interactions with the spores. The researchers hypothesized the following: amine oxides would to be able to disrupt ionic interactions and other non-covalent interactions found in the coat due to its high dipole moment on the nitrogen and oxygen atoms; chaotropic agents would disrupt salt bridges and hydrophobic interactions resulting in protein unfolding; salts would break apart salt bridges and alter the membrane structure by osmotic pressure changes; and lastly, copper compounds are also salts, but were specifically identified and tested due to their bactericidal activity. Alas, none of these compounds showed any effect on spore structure or viability.

2. Increased susceptibilities to mild killing treatments

Previous studies have shown that the spore cortex, coat, and exosporium are required, in varying degrees, for resistance to heat and mild chemicals and large molecules, such as ethanol and lysozyme, respectively. This is due to the role these structures play in maintaining the dehydrated state of the spore as well as just being a physical barrier. The goal for treating spores with chemicals from a variety of classes and reactivities was to identify one or more chemistries that would create a gap, or break(s), in one or more of these structures. This damage can make the spores more penetrable by large molecules or even make them lose their dehydration state, making them more sensitive to heat and chemical treatments. Over the previous period of performances, several chemicals were identified that damaged the coat, exosporium, the cortex, or any combination of them.

The next step was to determine whether treatment with these damaging chemicals made the spores more susceptible to treatment with ethanol (70% and 100%), lysozyme, or heat; treatments that are effective against other, less resistant cell types. Ethanol is a standard decontaminating reagent used in many laboratories. Typically used as a 70% solution, it can be quite effective against vegetative bacteria and viruses. However, spores are very resistant to treatment with ethanol. Both 70% and 100% ethanol were tested. Lysozyme is a protein that degrades peptidoglycan, also known as the bacterial cell wall. Spores have a cell wall like structure known as the cortex that is very susceptible to degradation by lysozyme. However, an intact spore is resistant to lysozyme because the cortex is protected by the coat and exosporium, which are outside the cortex. These two structures act as sieves and block large molecules like lysozyme from penetrating the spore and reaching the cortex. Breaks in these structures may allow lysozyme to reach the cortex thereby making the spore more susceptible to lysozyme activity. Finally, spores are also inherently resistant to wet heat and this can be a measure of spore core dehydration. If the spores lose their dehydration state, they become more sensitive to wet heat. Typically spore heat resistance is tested by treating them to 65^{o}C for 30 minutes, a treatment fully survived by intact spores. Spores of *B. anthracis* Sterne were tested for increased susceptibility to these reagents following treatment by the chemicals that produced notable structural damage as noted in the above list. This test was conducted first by treating 5 x 10^7 spores with each chemical under conditions that had previously caused the structural damage. For all chemicals except DTT, the spores were incubated for 60 minutes at 25°C. For DTT, the spores were incubated for 24 hours at 45°C. The spores were then washed and resuspended in 1 ml 0.1% tween 80 and 0.2 ml (1 x 10^7) was moved to each of 4 fresh tubes and pelleted. One pellet was resuspended in 1 ml 0.1% tween 80 and placed in a 65°C water bath for 30 minutes; another pellet was resuspended in 1 ml 100% ethanol and another pellet in 1 ml 70% ethanol and left at room temperature for 15 minutes; the fourth pellet was resuspended in 0.05M Tris HCl pH 7.8 containing 0.5 - 25 mg/ml lysozyme and incubated 37°C for 15 minutes. 0.8 ml 0.1% tween 80 was added to the original tube to make similar spore concentrations and used as a chemical treated viability control. Table 2 shows survivability of chemically treated spores to the mild treatments listed above:

Table 2. Viability of Spores after Chemical Treatment

Chemical Treatment	Viability			
	Untreated	**70% Ethanol**	**Lysozyme**	**65°C, 30 min**
Untreated	6.0×10^6	5.8×10^6	7.9×10^6	6.1×10^6
0.5 M Acetic Acid	5.0×10^6	6.7×10^6	6.3×10^6	6.7×10^6
0.5 M Hydrochloric Acid	8.0×10^5	1.5×10^4	1.2×10^6	8.7×10^5
1% Sodium Dodecyl Sulfate	5.1×10^6	3.4×10^6	4.7×10^6	6.3×10^6
0.5 M Dithiothretiol (DTT) (45°C, 24 hr)	7.8×10^6	6.8×10^6	6.6×10^6	6.4×10^6
Benzyl Mercaptan	8.6×10^6	4.9×10^6	1.7×10^6	8.6×10^6
1,4-Benzenedithiol	8.7×10^6	[a]	6.0×10^6	8.2×10^6
Thiourea	1.1×10^7	2.0×10^7	9.1×10^6	1.0×10^7
5% Sodium thiosulfate	5.8×10^6	4.6×10^6	5.3×10^6	6.2×10^6
2,5- Dichlorophenol	4.2×10^6	[a]	4.0×10^6	2.9×10^6
1-Phenoxy-2-propanol	1.05×10^7	5.9×10^6	1.81×10^6	8.7×10^6
N, N-dimethylformamide (neat)	2.8×10^6	3.1×10^6	3.2×10^6	2.8×10^6
2-Phenoxyethanol (neat)	1.2×10^6	5.4×10^6	5.9×10^6	3.3×10^6
1 M 1-Ethyl-3-Methylimidazolium Bis(trifluoromethylsulfonyl)imide	5.0×10^5	6.5×10^5	7.2×10^5	6.3×10^5
1 M 1-Ethyl-3-Methylimidazolium 1,1,2,2-Tetrafluoroethanesulfonate	5.0×10^5	6.5×10^5	7.2×10^5	6.3×10^5

[a] This was not completed as the chemical was dissolved in 100% Ethanol.

As evidenced by the table, the structural damage caused by each of these chemicals was not sufficient to make the spores more susceptible to these mild treatments tested. This is surprising for the lysozyme treatment since there were noticeably large gaps in the coat and exosporium following several chemical treatments. This suggests that 1) there is a matrix present between the coat and exosporium that remains intact sufficiently to prevent migration of lysozyme in the time allowed and 2) that there is sufficient inner coat material surrounding the cortex that will not allow passage of the lysozyme to the cortex. Although the second hypothesis is more plausible, the first cannot be ruled out since this matrix is not visualized by our TEM preparation method nor is its makeup or density known.

3. Overall Conclusions:

Spores are highly resistant to many different chemical treatments. Several classes of compounds have been tested against the spores including: acids, amine oxides, aromatic/organic compounds, chaotropic agents, copper compounds, ionic liquids, oxidizing agents, reducing agents, salts, and surfactants. Of all of these classes, reducing agents, oxidizing agents, and acids were shown to have the greatest effect on the spores. Acids caused the most severe damage to the spores, resulting in several breakages and some caused leaking of the core. Oxidizing agents in particular PES-solid Dahlgren Decon^{TM} caused damage to several structures. Reducing agents had various effects; some reducing agents cleaved layers of the spore exterior structures, while others caused disassociation between the layers. This suggests that disulfide bonds are integral in the spore exterior. However, even if these bonds are broken the spore still retains viability suggesting that these bonds are not critical for survival.

Acids and oxidizing agents had the greatest effect on viability with several logs of kill. Unfortunately the reducing agents showed very little effect on spore viability despite large structural changes. This study demonstrates just how resilient spores are to a wide range of chemicals and even when structurally damaged they still retain the ability to germinate. Clearly this study shows that very few chemicals significantly damage the coat and even fewer affect spore viability. The reason for the spore's resistance to many of the chemicals tested is poorly understood. A major limiting factor in this study is that the structural make-up and interactions within the spore exosporium, coat, and cortex is not understood. Another limiting factor is the ability to detect changes; TEM provides high resolution to gather detailed information, however small intermolecular damages may still not be visualized. These results reinforce the idea that the coat is a highly protective and selective barrier that prevents most chemicals from reaching and reacting with core components. The spore has also shown resilience in viability as the spore is able to survive despite damage to some of the spore's exterior structures. While this study has not successfully identified a mild chemical treatment to kill or make spores more susceptible to other mild treatments, it has shown just how effective the spore's exterior is in mitigating spore damage. Even with no mild decontamination solution identified, a stepping stone has been provided for future studies. Suggestions for future studies and directions are in the next section.

4. Future Studies:

This project has resulted in more questions than answers. A lot of questions have been generated on why the difference seen with reducing agents. Another set of questions is on the 3-D structure of the spore's exterior and the resilience of the spore despite observed structural damage. Most importantly the question of identifying a mild chemical treatment for spores still remains.

The different results for each of the reducing agents has left the door open to future studies. The question remains as to what role hydrophobicity, size, and redox potential play in selectivity and the observed reactions. Also, it is of interest to learn if the spore uses these disulfide bonds as the natural way to germinate and break out of the coat and exosporium. This will hopefully aid in learning more about spore structure.

The 3-D structure and resilience of the spore should be studied in more detail, particularly how the spore maintains viability even when damaged. New techniques should be developed to better detect damages that have occurred in order to get a bigger picture. Techniques such as GC-MS, LC-MS, and SDS-PAGE may greatly contribute to the overall understanding of a spore structure by giving more information on the damage occurring to the spore upon treatment with various chemicals. This will hopefully answer the questions on how the spore is so resilient as one can more clearly quantify the damage that has occurred.

To aid in determining a mild decontamination methods, future studies can explore other classes of compounds including: bases, germinant mimicking compounds, metals, permaganates, and more oxidizing agents and reducing agents. Also, combination treatments should be considered especially combination treatments that affect different regions of the spore. For example, combining DTT (affects exosporium) and thiourea (affects coat and cortex) which may help render the spore very liable and susceptible to mild treatments. This increased sensitivity may be seen when combining chemicals with the enzyme based treatments.

B. Developing a degradative enzyme-containing formulation that kills *B. anthracis* spores.

In this section, efforts are described to identify a gentle degradative enzyme-containing formulation that will kill *B. anthracis* spores. We describe our progress in achieving this goal, culminating in the final formulation that routinely reduces spore viability by over 5 orders of magnitude.

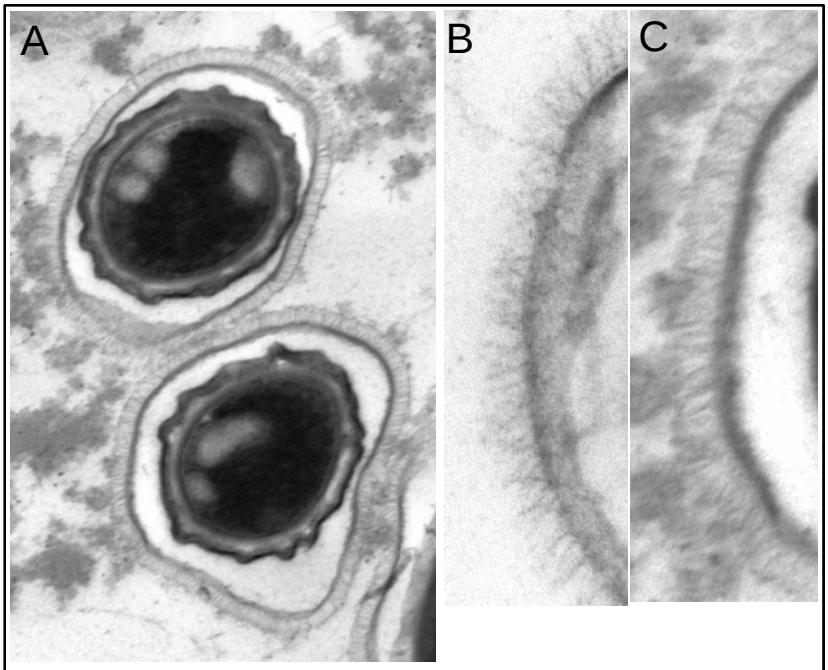

Fig. 9. Effect of papain treatment on *B. anthracis* Sterne strain spores. Papain-treated (A, C) or untreated (B) were imaged by thin section electron microscopy (see fig. 1). Spores (A) or magnified images showing only arcs of exosporium (B, C) are shown. Papain treatment causes the usually distinct fibers of the nap to appear as a continuous structure. Papain was used as described in the text.

This work proceeded in essentially three stages: 1) Identification of degradative commercial enzyme preparations that kill *B. anthracis* spores, 2) analysis of the effects on spore killing of addition of germinants to enzyme preparations, and identification of an optimal formulation and 3) analysis of the mechanism of enhancement of killing by germinants. Each of these stages is described in a separate section.

1. Identification of degradative commercial enzyme preparations that kill *B. anthracis* spores

a. Identification of a protease that breaches the coat and characterization of the role of the exosporium in defense against enzyme attack. In initial experiments, a protease was sought that can degrade the coat specifically since, in contrast to the exosporium, the existing literature argues that the coat provides the key resistance properties that make the spore invulnerable to mild decontamination. Therefore, proteases were applied to spores lacking the exosporium (because they harbor a mutation in the gene *cotE*) as well as intact spores. In our initial experiments, enzyme-treated spores were analyzed by electron microscopy (EM) so that we would detect any significant effect of a protease.

Commercial preparations of trypsin and papain were tested. It was found that while trypsin had no detectable effect on wild type or *cotE* mutant spores, papain had readily discernable and distinct effects on wild type and *cotE* mutant spores. After papain treatment (2 or 10 mg/ml, depending on the experiment (Fisher Scientific Corp.), in 1 mM EDTA, 5 mM L-cysteine, 0.07 mM β-mercaptoethanol, for 60' at 65^{o}C) wild type spores showed a morphological change on the

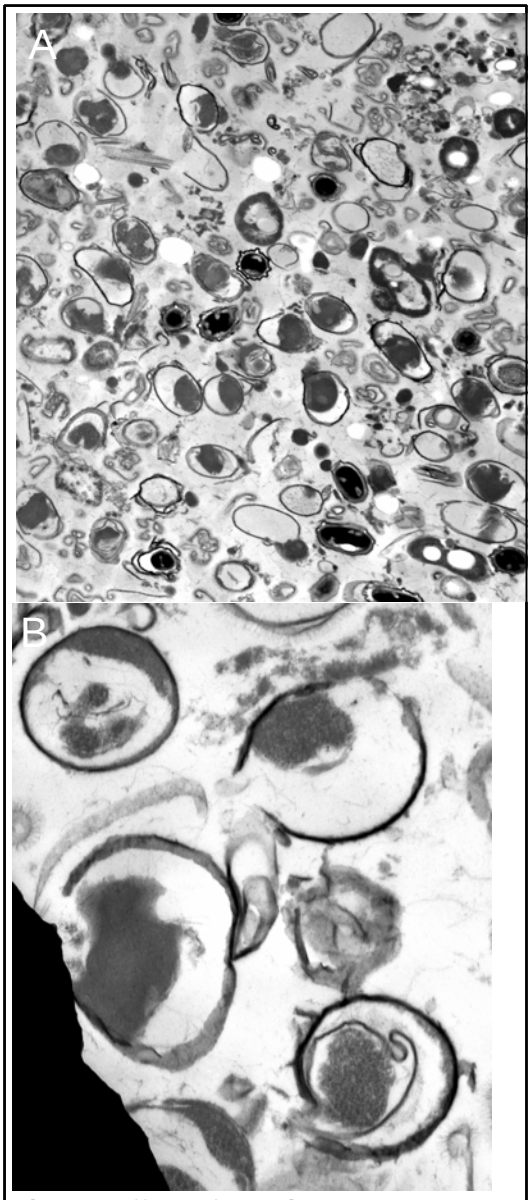

Fig. 10. Effect of papain treatment on *B. anthracis* Sterne strain spores lacking the exosporium due to a mutation in *cotE* A field (A) or an enlargement of just four (B) papain-treated spores. Papain treatment appears to result in breakage of the coat in at least one location and disappearance of the cortex. In the spores in (B), only the remaining coat and core cytoplasm are detectable after papain treatment. Papain was used as described in the text.

hair-like projections (or nap) on the exosporium surface (Fig. 9). The coat appears unaffected. This effect is consistent with the possibility that glycosylation of at least some exosporium surface proteins interferes with proteolysis (see below). In *cotE* mutant spores, however, papain treatment results in breakage of the coat, the absence of any detectable cortex, and loss of integrity of the core, which frequently appears to be "leaking" through the broken coat shards (Fig. 10B). While these findings are consistent with coat protein proteolysis (as expected), the severity of the damage (and, in particular, the absence of the cortex, which cannot be degraded by papain) raises the possibility that the coat is not, in fact, the target of papain in this experiment. To resolve this, two key questions were addressed: 1) are coat proteins actually targeted by papain during papain treatment of spores and 2) is the apparent papain-mediated degradation of the cortex the result of CwlJ, a cortex-lytic enzyme associated with the coat whose action, therefore, is possibly activated by coat protein proteolysis. The data presented so far suggest that the exosporium has a significant role in protection against degradative enzymes.

i. Coat proteins are degraded by papain during treatment of the spore. To learn whether coat proteins are degraded by papain, we harvested spores and shards of coat from papain-treated spores, extracted coat proteins by our standard methods, and analyzed protein degradation by SDS-PAGE. In summary, these experiments demonstrated that coat proteins are targeted by papain and that most if not all coat protein degradation occurs within about 2 minutes (data not shown). The data also established that the enzyme can be diluted by about 125-fold before any reduction in proteolysis is detectable (data not shown).

b. A formulation containing pronase, pancreatin and papain kills spores. Since our previous period data indicated that a papain preparation digests the coat and severely damages other spore structures, but does not penetrate the exosporium, it was important to achieve enzyme digestion conditions that would breach the exosporium. This was initially achieved with a cocktail of three commercial enzyme preparations: pronase (Roche, 10 mg/ml), pancreatin (Sigma, 10 mg/ml) and papain (Arcos Organics, 10 mg/ml), a treatment we refer to as P3. The usual reaction condition is a one hour treatment at 40^{o}C. It was found that individual or binary combinations of these enzymes did result in significant levels of damage, with the exception of papain and pancreatin which, together, resulted in ~1 log loss of viability.

Light microscopic analysis of P3-treated spores showed that about 90% of the spores were severely damaged. As for analysis of chemicals, the most direct way to assess the effect of an enzyme preparation on spores is by TEM. However, also as for chemicals, spore killing by an enzyme preparation is a very useful proxy for damage. Because the number of spores damaged is likely to be greater than the number of spores actually killed, it should be noted that using killing as a proxy for damage has the potential to underestimate the number of damaged spores.
It was found that P3-treated spores were reduced in viability by about 10^{2}-fold. Consistent with this, TEM analysis showed that about 90% of P3-treated spores had significant damage (data not shown). Importantly, damage to the exosporium was evident. The main conclusion from this experiment is that the P3 reaction conditions breach the exosporium.

It is important to note that these commercial enzyme preparations (as well as the others, discussed below) are not pure. Most if not all of them possess additional, often unidentified, enzymatic activities. The presence of these additional activities does not reduce their utility to our work. However, it does mean that we do not necessarily know which enzymatic activities in a preparation are required for the detected effect on the spore. This issue is discussed in more detail below.

c. An optimized treatment regimen with pronase, pancreatin and papain that results in greater levels of damage and killing. The results just cited demonstrate successful killing of intact spores by enzymes. However, since only 90% of spores were killed (and, at least at the resolution of TEM, damaged), it seemed plausible that our reaction conditions could be optimized. To achieve this, a variety of parameters were varied, many of which had no effect on improvement of spore killing or damage, and others are still being analyzed (see section 3, below). However, it was found that the application of the P3 treatment three times in succession (one hour each, at 40°C), which we refer to as 3P3, results in about 10^3-fold killing. No intact spores are visible by in 3P3-treated cultures by TEM. Instead, broken shards of exosporium and coat are abundant. There is no evidence of any forespores, or forespore-like structures (Fig. 11). There are, however, remnants of the forespore associated with exosporium and coat shards, in many cases. This is consistent with the previous results indicating that a peptidoglycanase activity in the papain preparation is able to significantly degrade spore peptidoglycan. In all cases the coat shards were empty; that is, there was no evidence of any forespore material inside the coat shards. In most cases, one or two breaks are visible in the otherwise contiguous coat shell. Where no break is evident, it can be inferred that the presence of a break above or below the plane of the section, since the enzymes could not reach the forespore region without a breach of the coat. Interestingly, we only rarely detected more than two breaks in a shard of coat. There is no definitive interpretation of this finding at present. However, it can be speculated that the breach points represent biochemically distinct regions of the coat that are especially sensitive to enzymatic degradation.

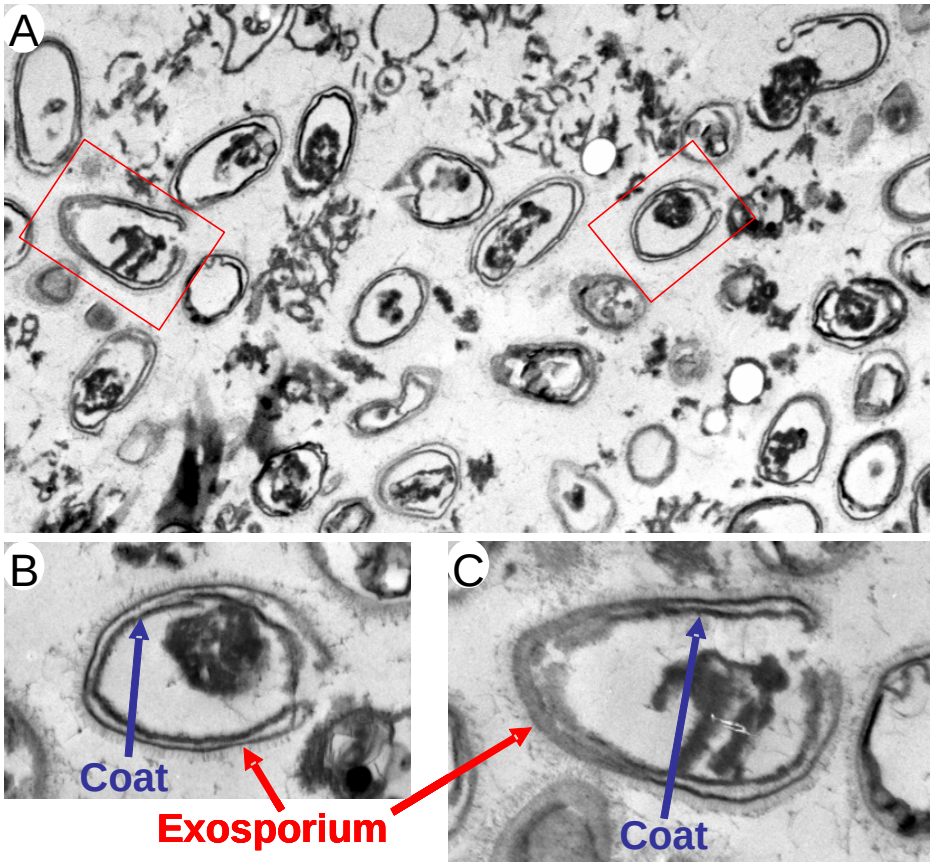

Figure 11. Electron micrographs of *B. anthracis* (Sterne strain) spores after treatment with 3P3. Individual digested spores (boxed in red) from the field in panel A are magnified in panels B and C, and the exosporium and coat in the shards are labeled.

As already discussed, measurements of killing are likely to under-represent the number of spores that are damaged. Therefore, it was hypothesized that among the approximately10^{-3} spores that survive 3P3 treatment, some might be damaged (albeit viable). To test this possibility, spores surviving 3P3 treatment were reacted with toluene, an organic solvent that does not kill intact spores but does kill spores bearing significant defects in spore protective structures. It was found that toluene treatment reduced viability by a further factor of ~10, indicating that 90% of the fraction (10^{-3}) of spores surviving 3P3 are significantly damaged in some as yet unknown manner. Importantly, toluene is not a good candidate chemical for our final agent defeat formulation, as toluene is a relatively harsh and toxic solvent. The purpose here is to use toluene solely to detect damaged spores among those surviving 3P3 treatment. Taken together with the data just described, these results indicate that, at a minimum, no more than approximately10^{-4} spores survive 3P3 treatment undamaged, and no more than approximately10^{-3} survive in a form that can be revived.

The data so far could under-represent the number of damaged spores for a number of reasons, including, for example, if the spores that are damaged are, nonetheless, resistant to toluene. This possibility might be appropriate to address in future experiments. However, a more likely possible mechanism of under-representation was addressed; the possibility that spores clumped after enzyme treatment and, as a result, became resistant to toluene. It is well established that in an aggregate, spores can be much more resistant to a wide range of stresses. To address this possibility, spores were first treated with 3P3, and then with either Tween-20 or Tween-80, detergents that should disaggregate spores that clumped during or after 3P3 treatment. Finally, toluene was applied, and the numbers of viable spores was measured. No significant change in viability after application of either detergent was detected. These data suggest that, to the degree that Tween 20 or Tween 80 can unclump spores under these conditions, spores are not protected from toluene treatment after 3P3 treatment due to clumping. These data are consistent with the interpretation that the toluene-sensitive spores that remain after 3P3 treatment are, indeed, damaged by 3P3, albeit to a lesser degree than those spores that are killed. A caveat on this interpretation is the possibility that the detergents we tested are unable to disaggregate at least some spores after 3P3 treatment. The importance of this caveat and the degree to which we should address it by treatment with additional detergents is still being assessed. Nonetheless, it is reasonable to suggest that aggregation is less likely to explain survival during toluene treatment.

The results discussed in the previous section strongly suggest that the exosporium is a significant barrier to digestion by enzymes including, in particular, papain. The finding that DTT causes breaches in the exosporium, reported above, gave us the opportunity to test this possibility in a more direct manner. To do this, spores were treated with 0.5 M DTT, the DTT removed by washing, and then reacted with papain. In this experiment, papain was used at its optimal reaction temperature of 65^{o}C (rather than 40^{o}C, as with P3 or 3P3). In the absence of DTT, effect of papain was detected. However, with DTT, 100-fold killing was measured. This result strongly supports the view that the exosporium is not significantly degraded by papain and that the exosporium also acts as a barrier excluding papain from contact with the coat. This barrier function is consistent with estimates of the porosity of the exosporium which suggest that papain is too large to penetrate the exosporium. The results just described were obtained with a 24 hour treatment with DTT. No effect of DTT was detected when treatment was for only 1 hour.

The data just described raised the possibility that DTT could enhance the ability of an enzyme formulation to damage spores. However, our data so far argue that this is unlikely, for several reasons. First, it was found that inclusion of even relatively low concentrations of DTT inhibit our enzyme formulation. It was also found that the ability of DTT to inhibit does not significantly diminish over long periods of time. As just an example of the results of a series of titration experiments, it was found that even after 24 hours, 100 mM DTT still inhibits the P3 reaction. Nonetheless, it was asked whether DTT could, in principle, enhance the P3 reaction (presumably, because some subset of spores retained their exosporium even after enzyme treatment). To do this, spores were treated with 0.5 M DTT for 24 hours, washed, and then treated with P3. No increase in spore killing was detected. This can be interpreted as evidence that P3-dependent spore damage is probably not limited by inefficient breaching of the exosporium, at least to the degree that can be compensated by DTT treatment under these conditions. Taken as a whole, the present data do not strongly indicate that DTT is unlikely to be useful adjunct to a future enzymatic formulation. Importantly, nonetheless, this result is highly meaningful to the basic science aspect of our tasking. Specifically, these result reveal that, most likely, redox events drive a key mechanistic event in exosporium shedding during germination.

d. An alternative formulation with amylase, trypsin, pronase, pancreatin and papain with significant killing. The 3P3 formulation just discussed was a promising initial enzymatic formulation for damaging spores. However, a concern with this approach is the requirement for multiple successive treatments with the enzyme cocktail. In a real-world application, a need for successive treatments might be suboptimal or even unacceptable. Therefore, as one approach to removing this requirement, the effects of incorporating additional enzymes into the P3 cocktail were investigated. This overall effort is described in detail later on. However, in an initial experiment, commercial preparations of amylase and trypsin (both from Sigma, respectively, each at 10 mg/ml) were incorporated into P3 and spores were treated for one hour at 40^{o}C. From this single round of treatment, spore viability was reduced by at least 2.5 logs. Using toluene, as already described, to assess the levels of damaged but viable spores, it was found that an additional 0.5 logs of killing occurred. Therefore, although some damaged but viable spores survive treatment with amylase, trypsin, pronase, pancreatin and papain, there are fewer such spores than after 3P3 treatment. Interestingly, in the case of amylase, trypsin, pronase, pancreatin and papain, three rounds of treatment resulted in ~10^3-fold killing, essentially the same as the level seen with 3P3 treatment. Although a superior formulation is described later on, these results above are included here to provide as complete a description of the effects of various enzymes as possible.

e. An alternative spore-killing formulation with amylase, trypsin and papain. It would be useful to have alternatives to at least some of the enzymes in the formulations just described. Given the importance of papain suggested by the experiments so far, amylase and trypsin were tested to determine whether, in combination with papain exosporium would be breached, and spores killed. It was found that treatment with an amylase, trypsin and papain cocktail (with each enzyme at 10 mg/ml) at 40^{o}C resulted in about a 5-fold level of killing. The treated spores have not been characterized spores treated by TEM.

f. Efforts to further optimize the treatment that were not productive. To explore whether the optimal number of repetitions of P3 treatment is three, the consequences of two and four

repetitions of P3 were also analyzed. It was found that two repetitions always resulted in ~10^2-fold killing, and four repetitions gave results similar to 3P3 (10^3-fold killing). This can be tentatively interpreted to mean that the improvement in killing and damage due to multiple P3 repetitions is due to a tendency of the enzyme cocktail to lose activity relatively rapidly and, in particular, for this to happen before all the spores have been digested at least to some degree. In this view, the addition of two additional treatments with P3 allows digestion of additional spores. If this is correct, then it is notable that four repetitions do not detectably improve digestion over three repetitions. The reason for this is not known, but it can be speculated that the small subset of spores surviving 3P3 treatment are physiologically distinct in a manner making them relatively insensitive to these enzymes. This possibility is not extraordinary; it is reasonable to posit, for example, that a very small subset of spores have assembled an especially thick coat due to fluctuations in the assembly process and, therefore, are hyper resistant.

The possibility that varying temperature, enzyme concentration (both by increasing the amount of enzyme and decreasing the numbers of spores), pH, or reaction time could increase the degree of damage or killing was also explored. These experiments suggested that altering these parameters is unlikely to improve the reaction.

An additional possible reason for a subset of undamaged spores already discussed; the formation of clumps during enzyme treatment (rather than afterwards, as already discussed). This phenomenon raises the possibility, therefore, that prior to enzymatic digestion, or as a consequence of digestion, a subset of spores form a protective aggregate. A possible way to address this, would be to perform the 3P3 regimen in the presence of a detergent. The challenge to performing this experiment is identifying conditions of detergent treatment that do not interfere with enzyme action.

Taken as a whole, the data described above suggest a working model that explains the degree of effect of 3P3 by positing that one or more enzymes in the P3 cocktail lose activity prior to completion of the reaction, necessitating three successive treatments and, in addition, spore populations possess a subset of physiologically distinct individuals that are resistant to these enzymes and/or form protective aggregates.

g. Analysis of alternative enzyme formulations that were not productive. A variety of additional commercially available enzyme preparations for damaging spores were tested (listed below). These experiments did not entirely exclude the possibility that one or more of these preparations could contribute to an optimal enzyme cocktail, because a fully exhaustive analysis would be very time consuming.

i. Bromolein. Bromolein is related to papain phylogenetically and functionally. However, its optimal temperature for activity is closer to room temperature than papain. Therefore, we tested whether it could substitute for papain. It was found that when bromolein was substituted for papain in several different reaction conditions, there was no readily detected spore damage.

ii. Lysozyme. Based on the TEM data indicating that the spore cortex is degraded by papain attack on spores missing the exosporium (data from our previous period of performance) and that fact that spores damaged by 3P3 have no visible cortex (Fig. 11), it can be strongly inferred that

the presence of a peptidoglycanase in the P3 formulation, that can attack the spore when the exosporium and coat are breached. To test whether killing could be improved by increasing the amount of peptidoglycanase activity in the formulation, 2 rounds of P3 treatment were performed, followed by treatment with lysozyme. The addition of lysozyme increased the level of killing by about 0.7 logs. It can be infered from this experiment that after two rounds of P3, some surviving spores have breaches in the coat and exosporium but intact cortexes. It should be noted that the addition of lysozyme did not result in killing above that seen with 3P3. Therefore, while lysozyme cannot apparently substitute for the final round of P3 in a 3P3 formulation, addition of lysozyme to the present formulations could enhance killing and/or damage.

iii. DNAse. Anecdotal evidence, and our own data from other ongoing work, suggest that the spore surface can be associated with a considerable amount of DNA. If so, then DNA could protect the exosporium or coat from digestion. To address this possibility, DNAse was applied to spores (under conditions judged in our other work to likely remove spore-associated DNA) prior to treatment with P3. There was no significant increase in spore damage.

iv. Pectinase, Cellulase and Viscozyme. A variety of enzyme preparations designed to digest plant material are commercially available. Three of the most readily available are Pectinase, Cellulase and Viscozyme, from Sigma. These products are crude preparations containing a variety of enzyme activities, some of which are not documented in the product literature. No significant damage to spores was detected when these enzymes were applied individually or together. Importantly, treatment with all three followed by papain did not result in any detectable spore killing. Therefore, it can be infered that these enzymes do not significantly breach the exosporium.

v. Amylase. The effect of adding a commercial preparation of amylase to our formulations was analyzed, not because it was suspected that amylase itself (which is a specific glycosidase) would have an effect, but rather because of the possibility that a contaminant in the amylase preparation could be useful. Application of a commercial amylase preparation (Sigma) alone did not result in spore damage nor, in combination with papain, pancreatin or pronase, did the addition of amylase result in greater damage than with any of those enzymes alone. However, by light microscopy, amylase, pronase and papain caused similar damage to what was detected with pancreatin and papain. Importantly, there was no killing. Successful use of amylase in more complex formulations is described above.

vi. Proteinase K. No spore damage was detected after treatment with proteinase K, with or without SDS (to which proteinase K is significantly insensitive and to which can facilitate proteinase K digestion by denaturation of substrate).

2. Analysis of the effects on spore killing of addition of germinants to enzyme preparations, and identification of an optimal formulation.

Table 3

Strain (all *B. anthracis* strains in the Sterne 34F2 background)	**Treatment condition**	**Logs Killed +/- SEM (standard error of the mean)**

B. anthracis	1hr RT 10mg/ml Papain	0.28 +/- 0.06
B. anthracis, *cotE*Δ::*kan*	1hr RT 10mg/ml Papain	1.0 +/- 0.1
B. subtilis, wildtype, PY79	1hr RT 10mg/ml Papain	0.96 +/- 0.04
B. anthracis	1hr RT 10mg/ml Papain + 100mM D-Alanine	0.04 +/- 0.08
B. anthracis	1hr RT 10mg/ml Papain + 100mM L-Alanine	2.2 +/- 0.2
B. anthracis	1hr RT 10mg/ml Papain + 100mM L-Alanine + 100mM D-Alanine	-0.02 +/- 0.1
B. anthracis	1hr RT 10mg/ml Papain + 50mM Inosine	2.6 +/- 0.1
B. anthracis	1hr RT 10mg/ml Papain + 100mM L-Alanine + 50mM Inosine	3.6 +/- 0.2
B. anthracis	1hr RT 10mg/ml Papain + 100mM D-Alanine + 100mM L-Alanine	1.5 +/- 0.1
B. anthracis	1hr RT 10mg/ml Papain + 50mM Inosine + 100mM D-Alanine	0.5 +/- 0.1
B. anthracis	1hr RT 10mg/ml Papain + 30mM CaDPA	1.3 +/- 0.2
B. anthracis	1hr RT 10mg/ml Papain + 60mM CaDPA	2.9 +/- 0.1
B. anthracis	1hr RT 10mg/ml Papain + 100mM L-Alanine + 50mM Inosine + 60mM CaDPA	4.1 +/- 0.1
B. anthracis	4.5hrs RT 10mg/ml Papain + 100mM L-Alanine + 50mM Inosine + 60mM CaDPA	5.0 +/- 0.2
B. anthracis	8hrs RT 10mg/ml Papain + 100mM L-Alanine + 50mM Inosine + 60mM CaDPA	5.3 +/- 0.2
B. anthracis	24hrs RT 10mg/ml Papain + 100mM L-Alanine + 50mM Inosine + 60mM CaDPA	5.14 +/- 0.01
B. anthracis	1hr RT No germinant followed by (->) 10min 37C 0.25mg/ml lysozyme	0.3 +/- 0.1
B. anthracis	1hr RT RT 100mM Alanine + 50mM Inosine -> 10min 37C 0.25mg/ml lysozyme	1.5 +/- 0.2
B. anthracis	1hr RT 60mM CaDPA -> 10min 37C 0.25mg/ml lysozyme	1.8 +/- 0.1
B. anthracis	1hr RT 60mM CaDPA + 100mM Alanine + 50mM Inosine -> 10min 37C 0.25mg/ml lysozyme	2.6 +/- 0.3
B. anthracis	4.5hrs RT No germinant -> 10min 37C 0.25mg/ml lysozyme	0.3 +/- 0.1
B. anthracis	4.5hrs RT 100mM Alanine + 50mM Inosine -> 10min 37C 0.25mg/ml lysozyme	1.4 +/- 0.2
B. anthracis	4.5hrs RT 60mM CaDPA -> 10min 37C 0.25mg/ml lysozyme	1.92 +/- 0.03
B. anthracis	4.5hrs RT 60mM CaDPA + 100mM Alanine + 50mM Inosine -> 10min 37C 0.25mg/ml lysozyme	2.3 +/- 0.1
B. anthracis	1hr RT No germinant -> 10min RT Toluene	0.6 +/- 0.1
B. anthracis	1hr RT RT 100mM Alanine + 50mM Inosine -> 10min RT Toluene	3.9 +/- 0.2
B. anthracis	1hr RT 60mM CaDPA -> 10min RT Toluene	3.08 +/- 0.04
B. anthracis	1hr RT 60mM CaDPA + 100mM Alanine + 50mM Inosine -> 10min RT Toluene	4.3 +/- 0.3
B. anthracis	4.5hrs RT No germinant -> 10min RT Toluene	0.3 +/- 0.4
B. anthracis	4.5hrs RT 100mM Alanine + 50mM Inosine -> 10min RT Toluene	4.4 +/- 0.4
B. anthracis	4.5hrs RT 60mM CaDPA -> 10min RT Toluene	3.0 +/- 0.3
B. anthracis	4.5hrs RT 60mM CaDPA + 100mM Alanine + 50mM Inosine -> 10min RT Toluene	4.3 +/- 0.3
B. anthracis	1hr RT 60mM CaDPA + 100mM Alanine + 50mM Inosine -> 1hr RT 10mg/ml Papain	3.67 +/- 0.2
B. anthracis	4.5hrs RT 60mM CaDPA + 100mM Alanine + 50mM Inosine -> 1hr RT 10mg/ml Papain	4.00 +/- 0.2

Buffer conditions using papain were in 40mM Phosphate and 25mM NaCl unless Ca^{2+}-DPA was present

Ca^{2+}-DPA solubilized in Tris at pH 7.4

Lysozyme buffer is 10mM Tris pH 8.0

In this part of the work, the major goal was to improve the action of the enzyme cocktail. In particular, it was hoped to increase the level of killing, reduce the numbers of enzymes in the cocktail, and eliminate the need for multiple applications to achieve maximal killing.

It was found that all these goals were achieved by the inclusion of germinants in the reaction. Strikingly and most importantly, the data show that the improvement in killing (which is over two orders of magnitude) is much greater than the efficiency of germination as typically measured in the laboratory (by monitoring rehydration of the spore core or the appearance of colonies after germination is complete). Investigating the effect of germinants on spore killing in more detail revealed a novel feature of germination: disassembly of the coat, which is a necessary but poorly understood step in germination, takes place at a much higher efficiency than spore core rehydration or other downstream events, including outgrowth. This finding implies that coat disassembly is triggered by an as yet unknown germinant-mediated signaling event. Although an effect of our enzyme formulation on the exosporium was not directly detected, it can nonetheless be inferred that this treatment renders the exosporium more porous because, to kill the spore, enzymes must reach the spore interior.

The results indicate that, with the addition of germinants, there is no requirement for multiple enzymes or multiple applications of the formulation. The present formulation uses a single application of papain. A wide variety of germinants and germinant combinations have been examined. These experiments showed that maximal killing (routinely over 10^5 logs) is achieved using Ca-DPA, inosine and alanine as germinants. Although, as would be expected, this combination provides the most germination we see in our experiments, the effect of these germinants on killing is greater than their effect in germination, consistent with the view that an as yet unidentified, germinant-dependent signaling mechanism triggers coat and exosporium disassembly.

a. Germinants improve spore killing by the papain preparation. It was reasoned that inducing germination could render spores more susceptible to killing by an enzyme-based formulation, because spore protective structures, including the exosporium, coat and cortex, are shed or degraded during the germination process. Therefore, *B. anthracis* spores were treated with 100mM L-alanine, 50mM inosine and 10mg/ml papain at room temperature for 60 minutes. This resulted in a 3.6 log reduction (+/- 0.2, standard error of the mean) in plating efficiency (Table 3, above).

This result raised the possibility that at least some of the effects of the papain preparation on spores in our previous experiments (i.e., without the addition of germinants) was due to the presence of germinants in the enzyme preparation. To test this possibility, two experiments were performed. First, our papain-containing preparation was placed at 65^{o}C for 24 hours, to inactivate papain and any other heat-labile enzymes. The heat-treated preparation was then added to *B. anthracis* spores and germination was measured by optical density. It was found that the heat-treated preparation induced only about a 3% change in the OD600 after 60 minutes (Fig. 12A). This result suggests that the papain preparation does not possess enough germinant, or the correct types of germinant, to induce efficient germination on its own. However, it does not

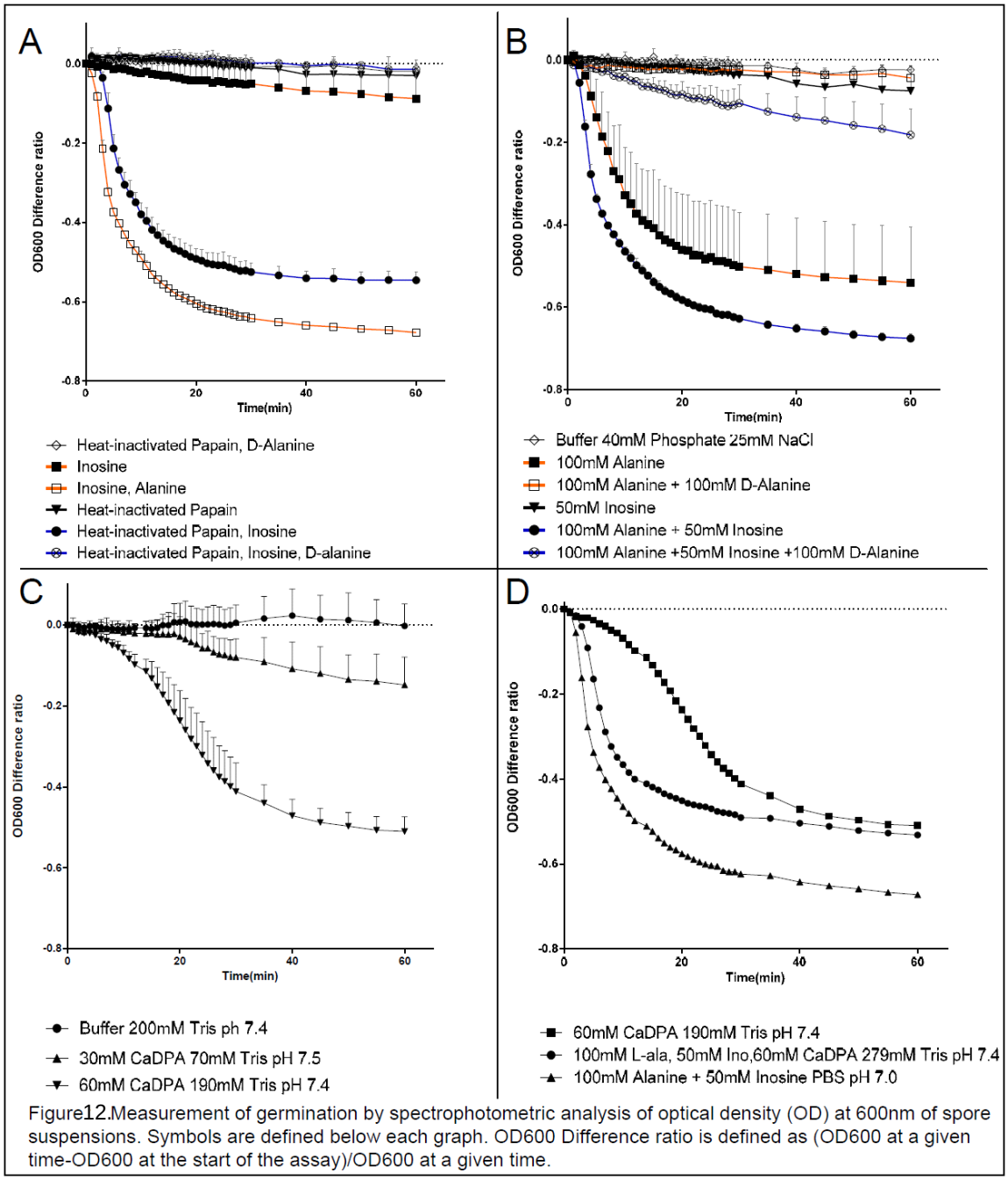

Figure12.Measurement of germination by spectrophotometric analysis of optical density (OD) at 600nm of spore suspensions. Symbols are defined below each graph. OD600 Difference ratio is defined as (OD600 at a given time-OD600 at the start of the assay)/OD600 at a given time.

exclude the presence of a molecule (such as L-alanine) that can act as a potent germinant (i.e., a cogerminant) in the presence of another molecule (which, in the case of L-alanine, could be inosine). Therefore, to address this possibility, a second experiment was performed in which the hypothesis that L-alanine is present in the papain preparation was tested. To do this, the effect of the addition of D-alanine to the formulation was analyzed, as this amino acid might competitively inhibit L-alanine-dependent germination, based on experiments in *B. subtilis*. The prediction was that, if L-alanine is present in the preparation, the addition of inosine after heat-treatment would result in significant germination, and the addition of D-alanine and inosine would result in very little germination.

Consistent with the predictions just described, the addition of inosine to heat-inactivated papain resulted in a 55% reduction in the OD600, while the addition of D-alanine to heat-inactivated papain resulted in no significant reduction in OD600 (Fig. 12A). Importantly, it was found that the addition of inosine and D-alanine reduced the OD600 nm by 1%, indicating that germination was significantly inhibited relative to the germination caused by heat-inactivated papain and inosine (Fig. 12B). These data can be interpreted as indicating that the papain preparation contains a small amount of germinant which, while insufficient to allow papain alone to cause significant killing, nonetheless is likely to facilitate the papain-dependent killing observed when exogenous germinant is added to the formulation.

It can be expected that other germinants would also enhance papain-mediated killing, and it was possible that some germinants might result in greater killing than we had measured so far. To address those possibilities, the effect of germinants other than L-alanine or inosine on killing by papain was measured. L-alanine or inosine + L-proline or L-tryptophan, germinant combinations well characterized in *B. anthracis*, were tested. The level of germination induced by each germinant (or germinant cocktail) varied, consistent with previous results in the literature (data not shown). Importantly, none of these germinant treatments resulted in greater killing by papain than did L-alanine and inosine.

b. Effect of the addition of Ca-DPA on spore killing by the formulation. To possibly further improve germination-facilitated killing, the effect on spore killing of the addition of Ca-DPA to L-alanine, inosine and papain was tested. This resulted in a significant increase in killing from a 3.6 log reduction (+/-0.2) to a 4.1 log reduction (+/- 0.1). Germination with Ca-DPA, L-alanine and inosine was not significantly greater than with only L- alanine and inosine (Fig. 12C, D). Therefore, it is unlikely that the increase in killing, due to the addition of Ca-DPA to a formulation containing L-alanine, inosine and papain, is due solely to an effect of Ca-DPA on germination. To further explore the mechanism by which Ca-DPA affects papain-dependent killing, the effect of Ca-DPA in the absence of L-alanine and inosine was analyzed. This resulted in a 2.9 log reduction (+/- 0.1) in plating efficiency. As expected from previous results in the literature, the 60 mM concentration of Ca-DPA resulted in efficient germination (Fig. 12). The ability of Ca-DPA to facilitate papain-dependent killing demonstrates that activation of the conventional nutrient germination receptors is not required for enhancement of papain-dependent killing.

To explore the effect of the duration of treatment on the level of killing, spores were treated with Ca-DPA, L-alanine, inosine and papain, as above, and allowed the reaction to proceed for 1, 4.5,

8, or 24 hours at room temperature (Table 3). The maximal level of killing was achieved after 4.5 hours with 5.0 logs killing (+/- 0.2), and there was no significant change in killing with more time. To address whether treating spores first with germinant and then with papain would improve killing and, in particular, would allow us to achieve maximal killing with a shorter duration of papain treatment, spores were first treated with germinants for 1 or 4.5 hours, removed the germinants, and then incubated with papain for 1 hour. In these experiments, killing was at least 10-fold less than when germinants were added together with papain. From this result, it can be inferred that germinant signaling is somehow more efficient in the presence of papain. Possibly, in this experiment, the action of papain results in increased exposure of receptor molecules to germinant.

To identify the spore structures damaged during killing by the addition of L-alanine, inosine, Ca-DPA and papain, TEM was performed on spores after this treatment. As expected from the addition of germinants, the cores of treated spores appeared largely granular (or punctate), but with small regions free of granules (Fig. 13A). This appearance is usually interpreted as evidence of core rehydration. No significant cortex is detectable. Also consistent with core rehydration is the apparently swollen state of the core, whose outer boundary in most cases is very close to the inner surface of the exosporium. In most spores, the coat does not encircle the spore. Instead, the coat appears as discontinuous pieces, disconnected from the forespore surface. In some spores, no electron dense layer consistent with a coat is detectable; in these spores, the coat may have been significantly degraded over its entire surface. Importantly, the exosporium appears unbroken. It should be emphasized that, although these spores appear somewhat similar to what would be expected from germinated spores, it can be inferred that most if not all of them are killed (i.e., unable to outgrow) based on the reduction in viability already discussed.

c. Analysis of the mechanism of germination-induced killing by the formulation. It was noted that the improvement of killing by papain due to germinant (to 4.1 log reduction) is far in excess of the level of core rehydration or outgrowth stimulated by germinant in our standard assays (as measured by the disappearance of refractility or outgrowth). This disparity suggested that germinants activate a process in the spore that renders it sensitive to papain, and that this activation occurs at a higher efficiency than core rehydration, as we usually measure it. One candidate process is the disassembly of the coat and/or exosporium. However, for outer layer disassembly to account for the disparity just noted, the efficiency of exosporium and/or coat disruption would have to occur at a much higher efficiency then core rehydration. Therefore, to measure coat and/or exosporium disassembly in response to germinants, *B. anthracis* spores were first germinated with either L-alanine and inosine, Ca-DPA alone, or L-alanine, inosine and Ca-DPA combined. All of these conditions induce core rehydration but do not permit outgrowth. It was then tested whether treatment with each germinant resulted in sensitivity to lysozyme or toluene. Lysozyme sensitivity is a well characterized proxy for impairment in spore outer layer assembly. Toluene sensitivity is also a widely used measure for impairment in spore protective structures, including but not necessarily limited to the coat. Strikingly, germination with L-alanine, inosine and Ca-DPA resulted in 2.6 logs (+/- 0.3) of killing by lysozyme and 4.3 logs (+/- 0.3) of killing by toluene (Table 3). Therefore, in response to germinants, a larger percentage of spores in a population are killed by lysozyme and toluene than become rehydrated. These data support the view that germinants enhance papain-dependent killing by activating a highly efficient disassembly of the spore's outer layers.

It is notable that treatment with germinants resulted in greater sensitivity to toluene than to lysozyme. The reason for this is unclear. However, it seems plausible that toluene might be better able to pass through minor breaches in the coat or exosporium than lysozyme, making toluene a more sensitive assay for outer layer disassembly.

To directly test the view that germination induces coat and/or exosporium disassembly, TEM was performed on spores after germination with L-alanine and inosine. It was found that all the spores appeared to have germinated to at least some extent, as all the spores had core morphologies consistent with significant rehydration (Fig. 13B). It was also noted that the spore shape was distorted from ovoid, possibly due to close packing. Most interestingly, almost none of the spores were fully encircled by a coat. Instead, the spore either appeared to lack a coat (as evidenced by the lack of any electron dense layer) or the coats were present as discontinuous shards, having apparently disassembled. In the latter case, it was common for the coat to appear to have separated into two separate hemispheres (see, for example, the single spore images in figure 13B). Possibly, the two hemispheres were driven apart by the force of cell volume expansion upon rehydration. Notably, the exosporium did not appear broken. Nonetheless, it can be inferred that the exosporium had, at a minimum, become relatively porous. This is based on our work in previous years indicating that the exosporium is a strong barrier that protects the spore interior against papain. Interestingly, in spite of the fact that most if not all the spores in figure 13A are killed (i.e., they are unable to outgrow based on the viability data discussed above), the spores in figures 13A and 13B appear very similar. This would be explained if the major targets of papain are inner membrane proteins with key roles in the resumption of metabolism during outgrowth, rather than the large scale degradation of specific cellular structures.

To identify the first point at which spores are likely start to disassemble the coat and/or exosporium in response to Ca-DPA, L-alanine and inosine, lysozyme sensitivity was measured to identify a relatively early time at which breaches in the outer layers appear. In a time course experiment, it was found that spores were significantly lysozyme sensitive after only 1 minute of exposure to germinants (data not shown). These data suggest that coat disassembly occurs relatively early in germination, likely around or before the cortex is cleaved and well before outgrowth.

3. Analyzing the mechanism of enhancement of killing by germinants.

a. Analyzing coat and exosporium disassembly during germination. The results so far

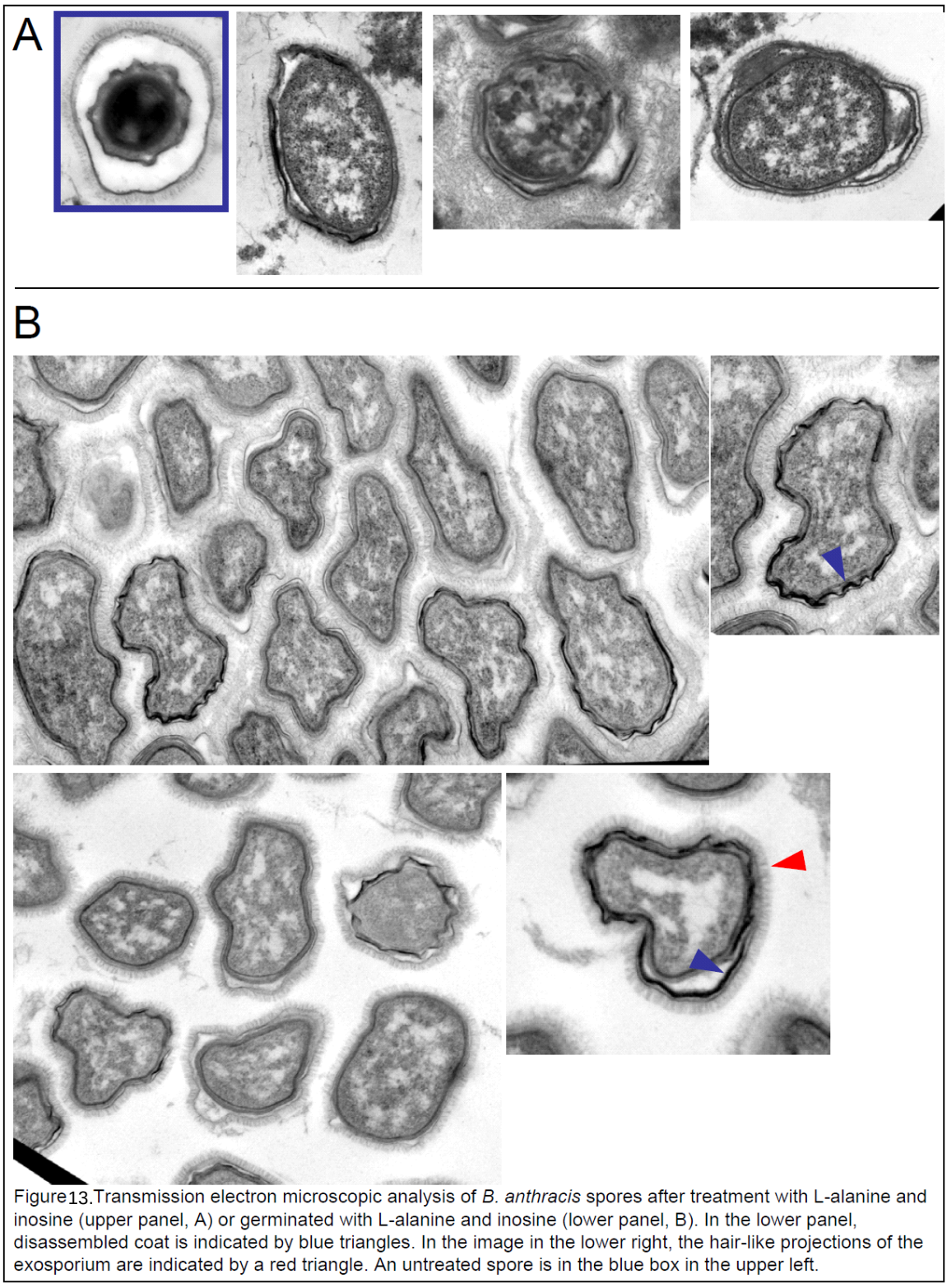

Figure13.Transmission electron microscopic analysis of *B. anthracis* spores after treatment with L-alanine and inosine (upper panel, A) or germinated with L-alanine and inosine (lower panel, B). In the lower panel, disassembled coat is indicated by blue triangles. In the image in the lower right, the hair-like projections of the exosporium are indicated by a red triangle. An untreated spore is in the blue box in the upper left.

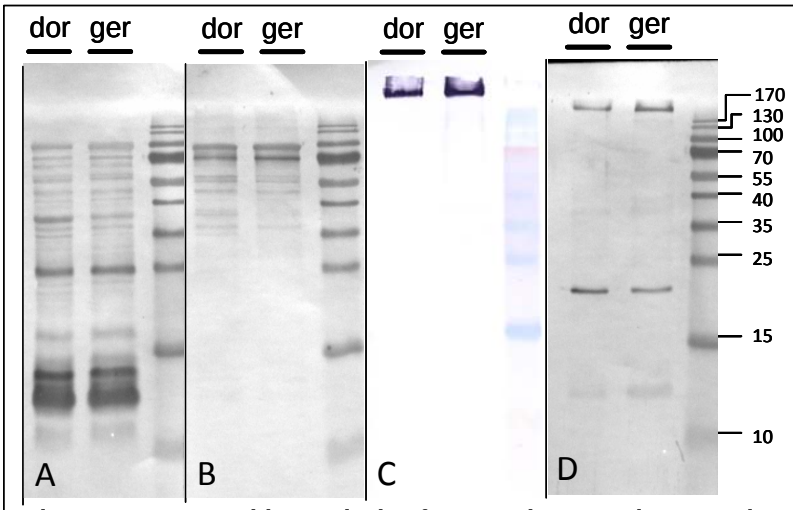

Figure 14. Western blot analysis of coat and exosporium proteins of dormant or germinated spores. Coat and exosporium proteins were extracted from dormant (dor) and germinated (ger) wild-type spores and then fractionated by SDS-PAGE, and subjected to Western blot analysis using the indicated antibodies and dilutions. A) anti-Cot α (1:50,000), B) anti-EA1 (1:1,000), C) anti-BclA (1:80,000), and D) anti-SOD (1:5,000). Molecular mass markers are on the right of each blot, and the masses are indicated on the extreme right.

strongly suggest that sensitivity to killing by papain (and, most likely, almost any degradative enzyme) using our optimized formulation, depends on the disassembly of the exosporium and coat. It was also shown explicitly that during germination the coat disassembles by breaking apart in to a small number of shards, and that the exosporium alters in a manner that allows entry of molecules that would otherwise be unable to penetrate the spore. Because TEM suggests that the exosporium remains a contiguous shell during germination, it can be inferred that germination causes a rearrangement in exosporium architecture (rather than a localized breach) with a concomitant increase in porosity. A reasonable speculation is that this structural rearrangement is an initial step in exosporium disassembly. Because of the inferred causal connection between coat and exosporium disassembly and killing, the possibility of optimizing germination by maximizing disassembly was explored. Possibly, even higher levels of germination and, therefore, improved killing might be achieved (see future directions). However, this approach will depend on signaling through the germination pathway and, therefore, will be subject to any intrinsic limitations in this pathway. Consequently, it was decided to characterize the process of coat and exosporium disassembly at the molecular level, with the goal of identifying mechanistic steps in the process that could be modulated by an improved killing formulation. Ultimately, the goal of this work is to identify reagents that could be added to the formulation that will increase disassembly, and therefore killing, to a degree beyond what can be achieved by addition of germinants.

As a first step in elucidating the mechanisms of coat and/or exosporium disassembly, it was hypothesized that this would involve biochemical changes in specific coat and/or exosporium proteins. It was speculated that any such proteins that did undergo changes might be targets of disassembly signals and/or proteins with key roles in coat and/or exosporium integrity. Therefore, to test this possibility, western blot analysis was used to compare spore protein extracts from dormant and germinated spores. It was expected that if a given protein was significantly altered during germination, its electrophoretic profile would change as well. To do this, a collection of antibodies against coat and exosporium proteins in our laboratory was used, including antibodies generated by us in the course of other work, generated by colleagues, and antibodies obtained from the BEI resource (NIAID) The antibodies were against Cot α ((Kim et al., 2004)), CotE ((Giorno et al., 2007)), BclA, BxpB, GerQ, p5303 and SOD (BEI resources ID #'s NR-9578, NR-12133, NR-10436, NR-12131 and NR-12129, respectively), and Alr, BclB,

EA1 and ExsK (the last 4 antibodies were generated by a collaboration between Lawrence Livermore National Laboratory and our laboratory, to support a separately (NIH) funded project).

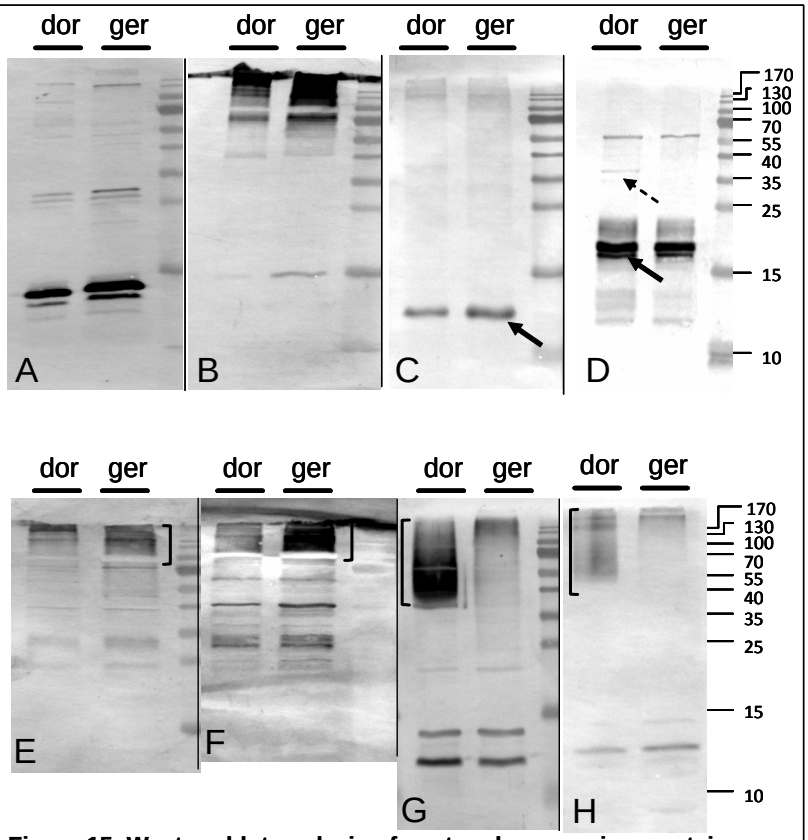

Figure 15. Western blot analysis of coat and exosporium proteins of dormant and germinated spores from wild type (A, B, C, D, E, F and G) or *cotE* mutant (H) spores. Western blot was performed as in figure 14. In each panel, dormant (dor) and germinated (ger) spores were reacted with the following antibodies at the indicated dilutions: A) anti-GerQ (1:3000), B) anti-BxpB (1:3000), C) anti-ExsK (1:50), D) anti-CotE (1:1000), E) anti-Alr (1:50), F) anti-BclB (1:50), G, H) anti-p5303 (1:3000). Large arrows point at bands at the predicted molecular masses for each protein. The dashed arrows in A and D indicate possible dimers. Brackets in panels E-H mark high molecular weight bands whose patterns differ between dormant and germinated spore extracts. Molecular mass markers are on the right of each blot, and the masses are indicated on the extreme right.

It was found that when using antibodies against the coat protein, Cot α, the exosporium protein BclA, the spore-associated superoxide dismutase (SOD) or the surface layer protein EA1 there were no detectible changes between the electrophoretic patterns from proteins from dormant or germinated spores (Fig. 14). However, application of antibodies against the coat proteins GerQ and CotE, and the exosporium proteins BclB, BxpB, ExsK, and Alr revealed modest but readily detected changes (Fig. 15). Anti-GerQ antibody recognized increased amounts of protein at bands ~34kD and ~15kD after germination (Fig 15A). Anti-BxpB detected a band at ~15kD and anti-ExsK detected a band at ~12kD that increased in intensity after germination (Fig. 15B and 15C respectively). Band at ~35kD that anti-CotE detected was lost after germination (Fig 15D). Anti-Alr as well as anti-BclB detected high molecular weight bands that increased in intensity after germination (Fig 15E and 15F). These results suggest changes in both coat and exosporium proteins. Most strikingly, dramatic changes were noted in a still poorly characterized protein called p5303 (Fig. 15). p5303 assembly is at least partially BclA-dependent and, therefore, likely on the exosporium surface (Cybulski et al., 2008). p5303 is also notable because its presence in a PA-containing vaccine adds to protection (Cybulski et al., 2008). We found that in dormant spores, a dense smear was detected at ~35kD (Fig. 15G). However, in germinated spores, the ~35 kD smear was no longer detected and a dense band at ~170 kD appeared. These data suggest that p5303 undergoes significant modification during germination. Interestingly, in *cotE* mutant spores, the overall levels of p5303 is lower than in wild type, and the electrophoretic pattern in dormant spores is somewhat

different than in wild type spores (Fig. 15H). Because the exosporium is not present in most *cotE* mutant spores (Cybulski et al., 2008), these data can be interpreted to imply that p5303 is present both on the exosporium surface as well as in the interspace (or coat surface) and, furthermore, that germination effects p5303 in both locations similarly. Taken as a whole, these western blot data further characterize a previously poorly recognized and completely uncharacterized step in germination, and identify proteins (especially p5303) with potentially key roles in coat and/or exosporium disassembly. Notably, a major gap in knowledge is the result of the lack of a p5303-deficient spore. Analysis of such spores could provide very useful information on germination and spore disassembly specifically.

b. Analyzing a possible signal for coat and/or exosporium disassembly. As a second approach to identifying steps in coat and/or exosporium disassembly that could be modulated in a future formulation, the signals that activate disassembly were investigated. Notably, there is no obvious molecular mechanism whereby the known events during germination (triggering, core rehydration) could signal events in the coat and exosporium.

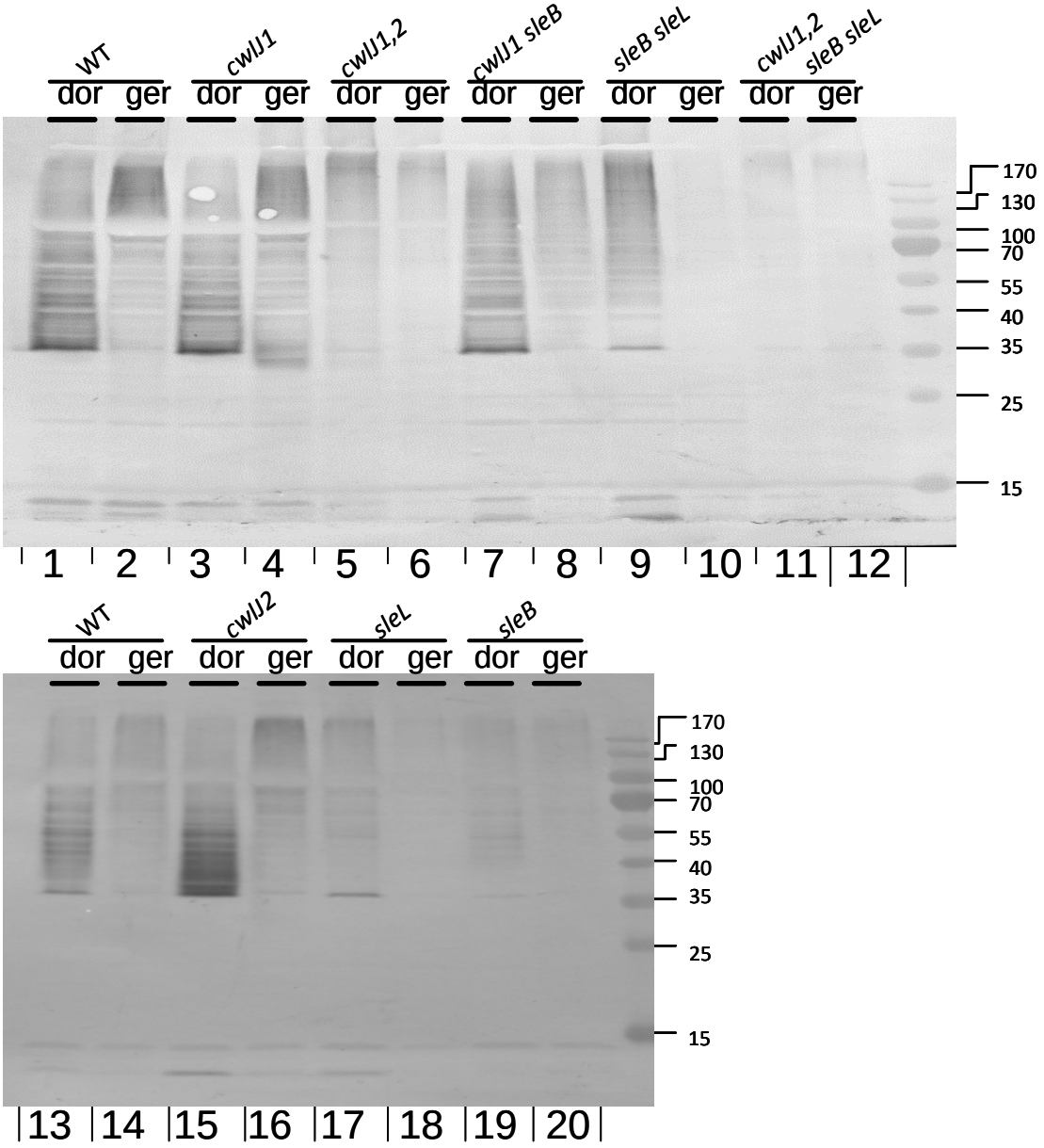

Figure 16. Western blot analysis of coat and exosporium proteins of dormant and germinated spores from wild type (lanes 1, 2, 13 and 14) and the following mutant strains: *cwlJ1* (lanes 3 and 4), *cwlJ2* (15 and 16), *sleL* (17 and 18), *sleB* (19 and 20), *cwlJ1 cwlJ2* (5 and 6) *cwlJ1 sleB* (7 and 8), *sleB sleL* (9 and 10) and *cwlJ1 cwlJ2 sleB sleL* (11 and 12). Western blot was performed as in figure 14 (except that a 12% gel was used) and anti-p5303 was applied at a 1:3000 dilution. Molecular masses are indicated on the extreme right.

Therefore, the hypothesis that disassembly is activated by the production of cortex lytic fragments that emanate from the spore core during germination was tested. This hypothesis arises from the now well-established sequence of events in the early-to-intermediate stages of germination (Heffron et al., 2010; Heffron et al., 2009; Setlow, 2003). Specifically, soon after germinants trigger core rehydration, the cortex is lysed by cortex lytic enzymes (CLE's). This allows the core to swell to the degree needed for the resumption of metabolism. As a byproduct of the reaction however, cortex fragments (that is, peptidoglycan fragments) are generated and released from the core. Based on these results, it was hypothesized that cortex lytic fragments stimulate coat and/or exosporium disassembly.

To test the hypothesis, *B. anthracis* strains were obtained from Dr. David Popham (Virginia Tech University) bearing mutations in various combinations of cortex lytic enzyme genes *sleB*, *sleL*, *cwlJ1* and *cwlJ2*, which have been well characterized in the Popham laboratory (Heffron et al., 2010; Heffron et al., 2009). Western blot analysis was then used to ask whether a given mutation prevented the already changes in p5303 during germination, documented in figure 16. An effect of a mutation on the p5303 electrophoretic pattern after germination would constitute evidence that the cortex lytic products produced by the corresponding gene might play a role in disassembly.

It was found that some mutations (*cwlJ1, cwl2*, and *cwlJ1 sleB*) did not affect the electrophoretic profiles (either of dormant or germinated spores) (Fig. 16, lanes 1-4, 7,8,15,16) of p5303. However, several of the mutation combinations resulted in a significant alteration in the dormant-spore profile (Fig. 16, lanes 5, 9, 11, 17 and 19). The electrophoretic profiles of *cwlJ1 cwlJ2* mutant spore extracts (whether from dormant or germinated spores) were distinct from those of wild type, dormant spores. Interestingly, the profile of *cwlJ1 cwlJ2* mutant spore extracts resembled those of wild type spores (Fig. 16, lanes 5 and 6). The western blot data suggest that *sleL* and *sleB* mutant spores possess lower levels of p5303 than wild-type spores. In addition, however, the migration of p5303 in *sleB sleL* mutant spores also differs from the migration in either dormant or germinated wild-type spores.

Taken as a whole, these data are consistent with the possibility that cortex lytic enzymes have a role in proper assembly of the structural components of the coat and/or exosporium. This, in turn, is consistent with previous results indicating that in *B. subtilis*, CwlJ and the SleL homologue YaaH are coat proteins (Bagyan and Setlow, 2002; Bauer et al., 1999; Imamura et al., 2009; Kim et al., 2006; Little and Driks, 2001). Unfortunately however, because these mutations effect the dormant spore protein profile, it is not possible to use these data to address our initial hypothesis, which remains plausible. Nonetheless, these data reveal important functions for CwlJ1 and SleL (and perhaps other CLEs) in *B. anthracis* coat assembly. These results will almost certainly provide useful information towards an improved understanding of the control of cortex lysis and coat disassembly during germination.

c. Analyzing spores that survive treatment. Possibly, insight could be gained into ways to improve killing if it was understood why some spores survive treatment with the formulation. Therefore, the possibility that spores that survive treatment are nonetheless damaged (at least to

some degree) and, therefore, sensitive to application of an additional harsh reagent was tested. The resulting data could reveal the nature of the damage sustained by the survivors and, as a result, something about the mechanism of survival. To address this, surviving spores were treated with toluene (exactly as was done to analyze spores surviving 3P3, above) and with DTT, also as above. Spore viability was decreased by no more than 0.3 logs by either treatment (data not shown). Therefore, it is likely that the surviving spores are not damaged in a manner that makes them sensitive to toluene or DTT. It can be inferred from these data that these spores have a relatively intact coat and exosporium and are not damaged in a manner that makes them overly sensitive to a redox reagent.

4. Future directions:

There are several aspects of the work described in this section that could usefully further analyzed in future studies. Some of these are listed below.

a. Using enzymes other than or in addition to papain. It is possible that we may achieve greater killing by using an enzyme other than (or in addition to) papain, in combination with germinants. Although a large number of enzymes have been already analyzed in our work so far, only a small subset of these were analyzed in combination with externally applied germinants.

b. Using peptidoglycan as a germinant. It was found that varying the formulation to optimize germination also optimized killing. Since peptidoglycan (PG) can serve as a germinant (Shah et al., 2008), it is reasonable to speculate that the addition of PG to our formulation will improve killing. Importantly, the ideal PG for stimulating germination in *B. anthracis* remains unknown.

c. Improve killing by understanding the failure to kill a subset of spores. It is not known why some spores fail to be killed in our current formulation. Possibly, spores that survive are relatively insensitive to germination, refractory to papain, or both. Plausibly, if these surviving spores could be harvested in sufficient yield, standard molecular and cellular methods could be used to analyze them and address these possibilities. To do this, a suggestion made to us by Dr. Peter Setlow was employed. First, a standard enzyme killing reaction was performed a, but using a very large volume of spores. After killing, intact spores were separated from digested (and therefore at least partially destroyed) spores by a Nycodenz gradient method. To ensure efficient separation of the digested spores from the intact spores, after enzyme treatment the protocol would then involve addition of SDS, to cause any spores that were successfully digested to float towards the top of the gradient. Unfortunately, after application of the Nycodenz gradient purification to digested spores, no phase bright spores were recovered in either the top or bottom gradient fraction. Using this method, very few spores were recovered. While this may be due to the use of too few spores, it is also possible that the behavior of digested spores was not as expected.

The optimal method to isolate the rare survivors remains unknown. However, one method that should be considered is fluorescence-activated cell sorting (FACS) (using any of several appropriate strains generated in our laboratory in the past, that produce fluorescently labeled spores) and cell fractionation techniques. Our previous work shows that FACS can isolate damaged spores for further experiments using forward vs. side-scatter. However, initial

calculations indicate that harvesting a sufficient amount of spores for down stream analyses will require a very long sort time. Therefore, this approach might not be practical unless a dedicated FACS facility is available.

d. Analysis of the mechanism of germination. Gentle decontamination of bacterial spores is an important need that has resisted a practical solution in spite of many decades of research. It can be inferred from this and from our own work identifying novel mechanisms of loss of resistance during germination, that part of the reason for a lack of progress is a failure to appreciate how our basic science knowledge gaps impact our ability to achieve practical technologies. Specifically, this suggests that an appropriate future direction is to further elucidate the mechanisms of disassembly of spore protective structures. Arguably, this is especially important, as fully disassembled spores should be essentially completely susceptible to an appropriate gentle killing formulation. Importantly, a key implication of our work is that without further facilitating disassembly, further improvement in killing might be impractical or effectively impossible.

Continuing to attempt to address the hypothesis that cortex lytic fragments signal disassembly is also worthy, because if true it would immediately provide a pathway to improving the formulation. It is also likely to be very useful to continue to characterize biochemical changes in the coat and exosporium during germination, to identify spore proteins involved specifically in disassembly and, therefore, which could be targeted in an improved formulation.

References

Bagyan, I., and Setlow, P. (2002). Localization of the cortex lytic enzyme CwlJ in spores of *Bacillus subtilis*. J Bacteriol *184*, 1219-1224.

Bauer, T., Little, S., Stöver, A. G., and Driks, A. (1999). Functional regions of the *Bacillus subtilis* spore coat morphogenetic protein CotE. J Bacteriol *181*, 7043-7051.

Cybulski, R. J., Jr., Sanz, P., McDaniel, D., Darnell, S., Bull, R. L., and O'Brien, A. D. (2008). Recombinant *Bacillus anthracis* spore proteins enhance protection of mice primed with suboptimal amounts of protective antigen. Vaccine *26*, 4927-4939.

Giorno, R., Bozue, J., Cote, C., Wenzel, T., Moody, K.-S., Ryan, M., Wang, R., Zielke, R., Maddock, J. M., Friedlander, A.*, et al.* (2007). Morphogenesis of the *Bacillus anthracis* spore. J Bacteriol *189*, 691-705.

Heffron, J. D., Lambert, E. A., Sherry, N., and Popham, D. L. (2010). Contributions of four cortex lytic enzymes to germination of *Bacillus anthracis* spores. J Bacteriol *192*, 763-770.

Heffron, J. D., Orsburn, B., and Popham, D. L. (2009). The Roles of Germination-Specific Lytic Enzymes CwlJ and SleB in *Bacillus anthracis*. J Bacteriol.

Imamura, D., Kuwana, R., Takamatsu, H., and Watabe, K. (2009). Localization of proteins to different layers and regions of *Bacillus subtilis* spore coats. J Bacteriol *192*, 518-524.

Kim, H., Hahn, M., Grabowski, P., McPherson, D. C., Wang, R., Ferguson, C., Eichenberger, P., and Driks, A. (2006). The *Bacillus subtilis* spore coat protein interaction network. Mol Microbiol *59*, 487-502.

Kim, H. S., Sherman, D., Johnson, F., and Aronson, A. I. (2004). Characterization of a major *Bacillus anthracis* spore coat protein and its role in spore inactivation. J Bacteriol *186*, 2413-2417.

Little, S., and Driks, A. (2001). Functional analysis of the *Bacillus subtilis* morphogenetic spore coat protein CotE. Mol Microbiol *42*, 1107-1120.

Setlow, P. (2003). Spore germination. Curr Opin Microbiol *6*, 550-556.

Shah, I. M., Laaberki, M. H., Popham, D. L., and Dworkin, J. (2008). A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. Cell *135*, 486-496.

DEPARTMENT OF DEFENSE

DEFENSE THREAT REDUCTION
AGENCY
8725 JOHN J. KINGMAN ROAD
STOP 6201
FORT BELVOIR, VA 22060
ATTN: H. MEEKS

DEFENSE TECHNICAL
INFORMATION CENTER
8725 JOHN J. KINGMAN ROAD,
SUITE 0944
FT. BELVOIR, VA 22060-6201
ATTN: DTIC/OCA

DEPARTMENT OF DEFENSE CONTRACTORS

QUANTERION SOLUTIONS, INC.
1680 TEXAS STREET, SE
KIRTLAND AFB, NM 87117-5669
ATTN: DTRIAC